University of Alberta

Structural-Functional Studies of *Helicobacter pylori* α1,3/4 Fucosyltransferases

by

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For my parents Wei Ji Ma and Pei Wen Zhou

ABSTRACT

The lipopolysaccharide of *Helicobacter pylori* contains fucosylated oligosaccharides on its O-antigens, predominantly Type II blood group antigens and less commonly Type I blood group antigens. Increasing evidence shows that Lewis antigens are not an absolute prerequisite for *H. pylori* colonization and adhesion to gastric epithelial cells. Instead, they only play a minor role in both processes. Nevertheless, their expression is often associated with severe gastric pathology. Besides, Lewis antigens can modulate the host immune responses through phase variation by interacting with dendritic cells, thereby promoting persistent infection.

Fucosyltransferases (FucTs) in *H. pylori* are responsible for the last steps in the synthesis of Lewis antigens. FucTs catalyze the transfer of L-fucose from guanosine diphosphate β-L-fucose to Type II (Galβ1,4GlcNAc) and Type I (Galβ1,3GlcNAc) structures on glycoconjugate acceptors at α1,2-, α1,3-, α1,4- or α1,6-linkages. α1,3/4 and α1,2 FucTs have been identified in *H. pylori*. To date, the knowledge of the structure-function of prokaryotic α1,3/4 FucTs, unlike their mammalian counterparts, is much less advanced. In this thesis, *H. pylori* α1,3/4 FucTs were functionally characterized by identifying the determinant for Type I acceptor specificity, mapping the minimal catalytic domain, kinetically characterizing purified enzyme, and examining the key polar groups in the acceptor substrates that are essential for recognition by *H. pylori* α1,3/4 FucT.

FucTs from *H. pylori* strains NCTC11639 and UA948 were chosen for study as the former was reported to have an exclusive α1,3 activity and the latter has a substantial level of both α1,3 and α1,4 activity. A highly divergent C-terminal segment of these two

FucTs was firstly identified by domain swapping as the molecular determinant for Type I acceptor specificity. Subsequently, data from site-directed mutagenesis indicated a single tyrosine residue in that region was essential. Moreover, only the C-terminus of *H. pylori* α1,3/4 FucTs can be truncated without significant loss of activity. For both Type II and Type I acceptors, the 6-OH of galactose is required for recognition by *H. pylori* α1,3/4 FucTs. In conclusion, our data demonstrated that, in spite of the very low sequence homology, *H. pylori* α1,3/4 FucTs share striking functional similarities with their mammalian counterparts.

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LIST OF ABBREVIATIONS

AIEX	anion exchange chromatography
BabA	Lewis B binding adhesin
bp	base pair
C1-β3GnT	core 1 β1,3-*N*-acetylglucosaminyltransfease
C2GnT-I	core 2 β1,3-*N*-acetylglucosaminyltransfease
cag PAI	*cag* pathogenecity island
CAZY	Carbohydrate-Active enZYme
cds	coding sequences
CE-LIF	capillary electrophoresis with laser-induced fluorescence detection
DC-SIGN	dendritic cell-specific ICAM-3-grabbing nonintegrin
DD-Hep	*D*-glycero-*D*-*manno*-heptose
ER	endosomal reticulum
FucT	fucosyltransferase
GDP-Fuc	GDP-fucose
GFP	green fluorescent protein
GlcNAc	*N*-acetylglucosamine
GT	glycosyltransferases
GTA	α1,3 *N*-acetylgalactosyltransferase A
GTB	α1,3 galactosyltransferase B
HCA	hydrophobic cluster analysis
H-Type I	2-fucosylated Type I
H-Type II	2-fucosylated Type II

IL-8	Interleukin-8
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl-β-*D*-thiogalactopyranoside
Kdo	3-deoxy-*D*-*manno*-octulosonic acid
LacNAc	Galβ1,4-*N*-acetylglucosamine
Le^{a}	Lewis A
Le^{b}	Lewis B
Le^{x}	Lewis X
Le^{y}	Lewis Y
LPS	lipopolysaccharide
MALT	mucosa-associated lymphoid tissue
mU	milliunit
NAP	neutrophil-activating protein
NEM	*N*-ethylmaleimide
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulphonyl fluoride
R-LPS	rough form of lipopolysaccharides
SabA	sialylic acid binding adhesin
SD	Shine-Dalgarno
SDS	sodium dodecyl sulfate
SHP-2	Src homology 2-containing protein-tyrosine phosphatase-2
sLe^{a}	3'-sialylated Lewis A
sLe^{x}	3'-sialylated Lewis X

S-LPS	smooth form of lipopolysaccharides
ST3Gal-I	α2,3 sialyltransferase I
ST6Gal-I	α2,6 sialyltransferase I
T4SS	Type IV Secretion System
TLC	thin layer chromatography
TMR	tetramethylrhodamine
TNF	tumor necrosis factor
Type I	Galβ1,3GlcNAc
Type II	Galβ1,4GlcNAc
Type II-R	Galβ1,4GlcNAc-O$(CH_2)_8CO_2CH_3$
Type I-R	Galβ1,3GlcNAc-O$(CH_2)_8CO_2CH_3$
VacA	vacuolating cytotoxin
WT	wild type

CHAPTER 1

General Introduction

1.1 *Helicobacter pylori* and Gastric Mucosa

Helicobacter pylori is a gram-negative, spiral shaped, micro-aerophilic bacterium that is unique among bacteria in its ability to colonize in the harsh environment of the human stomach. *H. pylori* may persist in the stomach for the lifetime of the host, suggesting they are well adapted to their biological niche. It is estimated that more than half of the world's population is infected by *H. pylori.* Although all individuals infected with *H. pylori* demonstrate gastric inflammation, the majority do not develop severe clinical symptoms. The presence of the bacterium is associated with an increased risk of developing peptic ulcers, and in rare cases leads to the development of gastric adenocarcinoma or mucosa-associated lymphoid tissue (MALT) lymphoma (Blaser and Berg, 2001; Suerbaum and Michetti, 2002).

H. pylori does not colonize the stomach evenly, but is restricted to the antrum and cardia (Schreiber *et al.*, 2004). Primarily an extracellular pathogen, *H. pylori* has the remarkable ability of being able to colonize the host gastric mucosa (Nakazawa *et al.*, 2003). Indeed, most *H. pylori* are found in the mucosal layer, preferentially at the cellular junctions (Amieva *et al.*, 2003) and a certain proportion tightly adhere to the surface of mucous cells (Noach *et al.*, 1994). Mucous cells secrete alkaline mucus that buffers the acidic environment of the gastric lumen (pH 2). As such, a pH gradient (pH 2 to nearly pH 7) is developed within the mucous layer (Schreiber *et al.*, 2004). Using advanced *in vivo* sampling technology, it was shown that *H. pylori* colonize a thin mucous layer, 0-25 μm directly adjacent to the surface of the stomach tissue in Mongolian gerbils, while the remaining 100 μm of the mucous layer is actually free of *H. pylori* (Schreiber *et al.*, 2004). In addition, the pH gradient along the mucous layer determines the orientation of *H. pylori* (Chen *et al.*, 1997; Schreiber *et al.*, 2004).

The gastric mucous cells secrete highly O-glycosylated molecules that form hydrated multimeric complexes, called mucins. In the human stomach, MUC5AC and MUC6 are the major secreted mucins. MUC5AC is produced by the surface and foveolar epithelium and by cells in the upper part of the gastric pits; whereas MUC6 is produced by the glands and confined to lower mucous neck cells and the antral glands cells (De Bolos *et al.*, 1995). Intestinal MUC2 may also appear in the gastric mucous layer when intestinal metaplasia and gastric cancer occurs. More than 99% of *H. pylori* are associated with either extracellular MUC5AC or the apical domain of MUC5AC producing cells (Van den Brink *et al.*, 2000). Recently it was reported that the failure of *H. pylori* to colonize the deeper portions of the gastric mucosa is due to the presence of multiple terminal α1,4-linked *N*-GlcNAc residues on the O-glycans of gastric gland mucosa (Kawakubo *et al.*, 2004). Such α1,4-linked *N*-GlcNAc structures, through an end-product inhibition mechanism, hinder the α-glucosyltransferase from transferring glucose from donor UDP-glucose to the C3 position of cholesterol at the α-linkage. As such the biosynthesis of cholesteryl-α-*D*-glucopyranoside was inhibited. The cholesteryl-α-*D*-glucopyranoside is a major cell wall component for *H. pylori* and is synthesized by taking the exogenous cholesterol as substrate, which cannot be intrinsically produced by *H. pylori*. Therefore, α1,4-linked *N*-GlcNAc structure functions as an antimicrobial to inhibit *H. pylori* survival in the deeper gastric glands (Kawakubo *et al.*, 2004).

In the human stomach, expression of MUC5AC is associated with Type I blood group structures, Lewis A (Le^a) and Lewis B (Le^b) (Fig. 1-1). On the other hand, MUC6

Fig. 1-1. Structures of Lewis blood group antigens. Gal: galactose, GlcNAc: *N*-acetylglucosamine, Fuc: fucose, GalNAc: *N*-acetylgalatosamine, Lewis X: Le^x, Lewis Y: Le^y, Lewis A: Le^a, Lewis B: Le^b.

Type II series

Type II (LacNAc)	$\mathrm{Gal\beta 1,4GlcNAc}$
Le^x^	$\mathrm{Gal\beta 1,4}\underset{\substack{\mid\,\alpha 1,3\\ \mathrm{Fuc}}}{\mathrm{GlcNAc}}$
H-Type II	$\underset{\substack{\mid\,\alpha 1,2\\ \mathrm{Fuc}}}{\mathrm{Gal}}\mathrm{\beta 1,4GlcNAc}$
Le^y^	$\underset{\substack{\mid\,\alpha 1,2\\ \mathrm{Fuc}}}{\mathrm{Gal}}\mathrm{\beta 1,4}\underset{\substack{\mid\,\alpha 1,3\\ \mathrm{Fuc}}}{\mathrm{GlcNAc}}$
Sialyl-Le^x^	$\underset{\substack{\mid\,\alpha 2,3\\ \text{sialic acid}}}{\mathrm{Gal}}\mathrm{\beta 1,4}\underset{\substack{\mid\,\alpha 1,3\\ \mathrm{Fuc}}}{\mathrm{GlcNAc}}$

Type I series

Type I (Lewis C)	$\mathrm{Gal\beta 1,3GlcNAc}$
Le^a^	$\mathrm{Gal\beta 1,3}\underset{\substack{\mid\,\alpha 1,4\\ \mathrm{Fuc}}}{\mathrm{GlcNAc}}$
H-Type I	$\underset{\substack{\mid\,\alpha 1,2\\ \mathrm{Fuc}}}{\mathrm{Gal}}\mathrm{\beta 1,3GlcNAc}$
Le^b^	$\underset{\substack{\mid\,\alpha 1,2\\ \mathrm{Fuc}}}{\mathrm{Gal}}\mathrm{\beta 1,3}\underset{\substack{\mid\,\alpha 1,4\\ \mathrm{Fuc}}}{\mathrm{GlcNAc}}$
Sialyl-Le^a^	$\underset{\substack{\mid\,\alpha 2,3\\ \text{sialic acid}}}{\mathrm{Gal}}\mathrm{\beta 1,3}\underset{\substack{\mid\,\alpha 1,4\\ \mathrm{Fuc}}}{\mathrm{GlcNAc}}$
A-blood antigen	$\mathrm{GalNAc}\overset{\alpha 1,3}{\overline{\quad}}\underset{\substack{\mid\,\alpha 1,2\\ \mathrm{Fuc}}}{\mathrm{Gal}}\mathrm{\beta 1,3GlcNAc}$
B-blood antigen	$\mathrm{Gal}\overset{\alpha 1,3}{\overline{\quad}}\underset{\substack{\mid\,\alpha 1,2\\ \mathrm{Fuc}}}{\mathrm{Gal}}\mathrm{\beta 1,3GlcNAc}$

expression is tightly associated with Type II antigens, Lewis X (Le^x) and Lewis Y (Le^y) (Fig. 1-1) (Cordon-Cardo *et al.*, 1986; Kobayashi *et al.*, 1993; Taylor *et al.*, 1998). As a result, Le^a and Le^b are mainly expressed on the surface epithelium, whereas Le^x and Le^y are predominantly expressed by the mucous, chief and parietal cells of the glands (Cordon-Cardo *et al.*, 1986; Kobayashi *et al.*, 1993; Taylor *et al.*, 1998), although the distinction is not always obvious (Murata *et al.*, 1992).

1.2 Virulence Factors of *Helicobacter pylori*

A number of virulence factors that aid in *H. pylori* infection have been studied. Several of the major virulence factors are discussed below. For a complete discussion of *H. pylori* virulence, the reader is directed to other recent reviews (Boneca, 2005; Dhar *et al.*, 2003; Gebert *et al.*, 2004; Hofman *et al.*, 2004; Rieder *et al.*, 2005; Sergeev *et al.*, 2004; Stoicov *et al.*, 2004).

1.2.1 Flagella

Motility is essential for *H. pylori* to initially colonize and chronically infect the gastric mucosa (Eaton *et al.*, 1992; Eaton *et al.*, 1996; Fimmel *et al.*, 1985; Ottemann and Lowenthal, 2002). The spiral shape of *H. pylori* and the polar flagella facilitate movement within the viscous mucous layer and allow *H. pylori* to escape from the extremely low pH within the gastric lumen. A recent study showed that in anesthetized Mongolian gerbils, *H. pylori* become irreversibly immotile in the lumen (pH 2-3) in less than 1 minute, whereas motility is maintained for 2 minutes at pH 4 and sustained for more than 15 minutes at pH 6 (Schreiber *et al.*, 2005).

1.2.2 Urease

H. pylori produces urease, a Ni^{2+}-containing cytoplasmic enzyme that converts urea into ammonia and carbon dioxide (Mobley *et al.*, 1991). The ammonia diffuses into the area surrounding the bacteria to increase the pH thereby promoting motility and survival. *H. pylori* possesses a mechanism to control the accessibility of the urea to the intracellular urease. Only under acidic conditions ($pH< 6.5$), are the urea channels present in the inner membrane of *H. pylori* open so that urea is transported and available for urease hydrolysis (Bury-Mone *et al.*, 2001; Weeks *et al.*, 2000).

1.2.3 *cag* Pathogenicity Island

A major virulence factor of *H. pylori* is the presence of a gene cluster of approximately 37 kb in size, called the *cag* pathogenicity island (*cag* PAI). The *cag* PAI is present in some strains either as a continuous unit or two split segments. In other strains, the *cag* PAI is absent. The *cag* PAI is flanked by 31 base direct repeats and is inserted into the glutamate racemase gene (Akopyants *et al.*, 1998), with a G+C content of 35% versus the 39% average for the *H. pylori* genome (Marshall *et al.*, 1998). This suggests that the *cag* PAI is an extrachromosomally derived element. The *cag* PAI contains 31 genes, several of which encode proteins that mediate the formation of a Type IV secretion system (T4SS) (Odenbreit *et al.*, 2000), which forms a needle-like apparatus attaching to the host gastric epithelial cells thereby translocating effector proteins into the host (Odenbreit *et al.*, 2000). One identified effector protein is CagA, which is encoded by the *cagA* gene located at the 3' end of the *cag* PAI and is considered a genetic marker of the *cag* PAI in *H. pylori*.

Once CagA is translocated by the T4SS into host cells, it is phosphorylated by a host kinase belonging to the c-Src/Lyn tyrosine kinase family (Stein *et al.*, 2000; Stein *et al.*, 2002). Phosphorylation occurs at a tyrosine residue within EPIYA motifs in the CagA protein (Puls *et al.*, 2002). The size of CagA from various *H. pylori* strains is distinctive due to the presence or absence of a 102-bp repeat in its central region (Higashi *et al.*, 2002a). For instance, the CagA is 128 kDa and 144 kDa when the 102-base pair (bp) repeat is absent or four 102-bp repeats are present, respectively. This 102-bp repeat region contains the EPIYA motif and the number of the EPIYA motifs also varies in different CagA proteins. It has been suggested that the level of CagA tyrosine phosphorylation is proportional to the number of C-terminal EPIYA motifs and to the degree of induced host inflammatory response (Higashi *et al.*, 2002a).

The translocated and phosphorylated CagA binds and activates the Src homology 2-containing protein-tyrosine phosphatase-2 (SHP-2) in the host cell (Higashi *et al.*, 2005), resulting in dephosphorylation of several unknown host proteins inlcluding one at 85 kDa and others at 120-130 kDa in size. These dephosphorylation events modify the host signal transduction pathway, inducing a growth factor-like response also known as the "humming-bird" phenotype and reorganization of the cytoskeleton to form a pedestal (Backert *et al.*, 2001; Higashi *et al.*, 2002b; Puls *et al.*, 2002). It is noted that only in some *H. pylori* strains is CagA also able to induce the production of interleukin-8 (IL-8) through activation of the transcription factor NF-κB in host gastric epithelial cells. This occurs via the Ras→Raf→Mek→Erk→NF-κB signaling pathway in a SHP-2- and a c-Met-independent manner (Brandt *et al.*, 2005). For those *H. pylori* strains where CagA fails to induce IL-8 induction, a *cagA* knockout disrupts cell elongation but not IL-8 expression. In contrast, knocking out *cagE*, the gene that encodes CagE an integral part of the T4SS, abolishes T4SS function and thus disrupts both host gastric epithelial cell elongation and IL-8 induction (Guillemin *et al.*, 2002).

1.2.4 Vacuolating Cytotoxin A

The vacuolating cytotoxin (VacA) is considered one of the major virulence factors of *H. pylori*, but shares no homology to any other known toxins (Cover, 1996). Under neutral pH conditions, VacA spontaneously assembles into large water-soluble flower-shaped oligomers, primarily with 12 or 14 monomeric subunits (Adrian *et al.*, 2002; Cover *et al.*, 1997; Lupetti *et al.*, 1996). Under acidic conditions (pH 5), the VacA oligomers break up into monomers and associate with the lipid bilayer forming membrane-associated hexameric anion-selective chloride channels (Kim *et al.*, 2004; Szabo *et al.*, 1999). After membrane insertion, VacA can be internalized and forms an anion-selective channel in endosomal membranes. The swelling of endosomal compartments leads to vacuole formation, thus blocking the intracellular late endosomal trafficking system (Czajkowsky *et al.*, 1999; Morbiato *et al.*, 2001; Szabo *et al.*, 1999).

vacA encodes a 140 kDa protein, which contains two divergent regions at the 5' terminus and the mid-region, designated as the s and m regions, respectively (Atherton *et al.*, 1995; McClain *et al.*, 2001). The VacA protein of the s2-type does not have vacuolating cytotoxicity (Ji *et al.*, 2000; Pagliaccia *et al.*, 1998). The presence of both *cag* PAI and VacA (s1, toxingenic type) in a *H. pylori* strain is associated epidemiologically with more severe diseases such as duodenitis, peptic ulcers, and gastric tumors (Covacci *et al.*, 1993; Xiang *et al.*, 1995). Strains possessing these two factors are classified as Type I strains whereas strains lacking of both determinants are classified as Type II strains (Censini *et al.*, 1996; Covacci *et al.*, 1993; Xiang *et al.*, 1995).

1.2.5 Adhesins

Adhesin molecules, enabling *H. pylori* to attach to gastric mucosa, initiate the first step of bacterial interaction with the host. Recently, *H. pylori* adhesins have drawn a lot of attention and many investigations have been carried out. Anti-adhesion treatment has also been proposed as an alternative therapy and has been tested in both animal models (Mysore *et al.*, 1999) and in clinical trials (Parente *et al.*, 2003).

1.2.5.1 Lewis b Binding Adhesin

Currently the Le^b binding adhesin (BabA) is believed to be the major adhesin involved in *H. pylori* adhesion. BabA binds to the Le^b tetrasaccharide and trisaccharide H-Type I structures (for chemical structures of Lewis blood antigens see Fig. 1-1). Both Le^b and H-Type I were found to be expressed on host gastric epithelial cells (Taylor *et al.*, 1998), with Le^b showing the higher affinity (Ilver *et al.*, 1998).

Three *bab* alleles, *babA1*, *babA2*, and *babB*, have been identified in *H. pylori* (Ilver *et al.*, 1998). *babA1* and *babA2* are almost identical except that *babA1* lacks a 10 bp fragment at its 5' end so that it is missing the translational initiation codon (Ilver *et al.*, 1998). Therefore, functional BabA is encoded only by *babA2*. *babB* encodes BabB, which shares nearly identical N-terminal and C-terminal domains with BabA but has a divergent middle region (Alm *et al.*, 1999). The function of BabB is at present unknown, and it has been shown that BabB cannot mediate the binding to Le^b on the gastric mucosa. (Ilver *et al.*, 1998).

Notably, BabA expression was found to be a dynamic process as the *babA1* gene can be turned on and off due to phase variation (Solnick *et al.*, 2004). Characterization of *H. pylori* strains recovered from rhesus macaques a few weeks after initial inoculation of the animals revealed that, *babA2* is either replaced by *babB*; or *babA2* is still present but not functional due to a frameshift mutation in the 5' poly(CT) tract (Solnick *et al.*, 2004). In either case, the recovered strains failed to bind the Le^b blood group antigen detected by ELISA. The on/off regulation of *babA2 in vivo* by phase variation might be one of the means *H. pylori* has evolved to adapt to the changing environment and achieve long-term infection (Haas *et al.*, 2002; Kimmel *et al.*, 2000; Solnick *et al.*, 2004).

The presence of *babA2* in *H. pylori* Type I clinical isolates (*cag* PAI^+ and $VacAs1^+$) is associated with increased bacterial colonization density, augmented IL-8 secretion, enhanced granulocyte infiltration (Rad *et al.*, 2002), severe histological modification of gastric tissue (i.e. intestinal metaplasia and atrophy), and is highly correlated to the prevalence of digestive ulcers and gastric adenocarcinoma (Prinz *et al.*, 2001). Interestingly, strains with the *cag* PAI^+ and $VacAs1^+$ but are *babA2*$^-$ do not correlate significantly with the severe histological changes (Prinz *et al.*, 2001). This suggests that the presence of all three virulence factors is a more accurate discriminator of disease outcome than the Type I/Type II classification (Prinz *et al.*, 2001).

1.2.5.2 Sialic Acid Binding Adhesin

In 2002, Mahdavi *et al.* discovered that *H. pylori* strain 17875 is able to adhere to *H. pylori*-infected, but not healthy gastric mucosa, even after inactivating both *babA1* and *babA2* (Mahdavi *et al.*, 2002). This prompted the discovery of another adhesin, sialic acid binding adhesin (SabA) and its ligand, the sialylated-dimeric-Le^x. The sialylated-dimeric-Le^x is rarely expressed on healthy human stomach but is heavily induced on both gastric surface epithelium and the deeper glands upon infection

(Mahdavi *et al.*, 2002). Compared to BabA-Le^b, the binding of SabA-sialyl-dimeric-Le^x is much weaker because of its relatively lower binding affinity (Mahdavi *et al.*, 2002).

1.2.5.3 Lewis X

The role of Le^x in adhesion was shown by studying *H. pylori* wild type (WT) strain NCTC11637 and its Le^x-negative mutants. The WT strain adheres avidly to the antrum of human stomach with tropic binding to the apical surface of mucosal epithelial cells and to cells of the gastric pits. In contrast, Le^x-negative mutants fail to adhere to gastric tissues (Edwards *et al.*, 2000). Remarkably, latex beads conjugated with synthetic Le^x also display tropic binding to the gastric epithelium (Edwards *et al.*, 2000). However, the ligand of Le^x has yet to be identified. Given the tropic binding pattern of Le^x to the gastric mucosa surface, the ligand is likely expressed exclusively by the surface mucous cells. Immunoelectron microscopy of both *H. pylori* and *H. pylori*-infected stomach biopsy demonstrates that the Le^x structure is present within the distinct adhesion pedestal and on both the *H. pylori* cell and gastric epithelium, suggesting the possibility of Le^x-Le^x interaction (Taylor *et al.*, 1998).

A recent study by Mahdavi *et al.* demonstrated that Le^x–mediated adhesion is not as significant as BabA-Le^b binding, but it is present at least in some *H. pylori* strains (Mahdavi *et al.*, 2003). For *H. pylori* strain 17875 (WT strain has a Le^x and Le^y negative phenotype), inactivation of *babA* dramatically reduced the adherence to healthy gastric mucosa, confirming BabA-Le^b mediated adhesion. But for *H. pylori* strain ATCC43504 that has a Le^x and Le^y positive phenotype, inactivation of *babA* only modestly reduced the adherence, indicating other adhesins such as Le^x are present. When BabA is functional and Le^x and/or Le^y expression were knocked out, bacterial binding ability to the gastric mucosa was not affected (Mahdavi *et al.*, 2003). This suggests that strong interaction between BabA and Le^b might have concealed $Le^{x/y}$-mediated adhesion. In the same study, *H. pylori* strain 11637 was chosen to study the role of Le^x-mediated adhesion under conditions where the BabA-Le^b interaction is absent as this strain is negative for Le^b binding. The ability of WT and Le^x-negative strains to adhere to 21 gastric tissues isolated from patients with diverse histopathological status was determined (Mahdavi *et al.*, 2003). WT and Le^x-negative *H. pylori* strains were found to attach to only 8 out of 21 gastric tissues. In 4 out of those 8 cases, the Le^x-negative mutants exhibited a significant reduction in adhesion compared to the WT strain (Mahdavi *et al.*, 2003). This revealed that Le^x played a minor role in adhesion and it is effective only in some strains.

1.2.5.4 Other Adhesins

In addition to BabA, SabA and Le^x, several other adhesins have been identified. For instance, neutrophil-activating protein (NAP) was reported to adhere to polymeric sulfated carbohydrates on high-molecular-weight salivary mucin and Le^x structure (Namavar *et al.*, 1998). Such binding is non-specific and can be greatly inhibited by dextran sulfate and DNA.

Two homologous proteins AlpA and AlpB possess a C-terminal region predicted to form a porin-like-β-barrel in the outer membrane. These two proteins also appear to be involved in adherence to both Kato III tissue culture cells and to human gastric tissue sections (Odenbreit *et al.*, 1999; Odenbreit *et al.*, 2002).

Under acidic conditions, *H. pylori* are also found to bind to mucins containing sialylated structures including sialyl-Le^x and sialyl-Type II (Fig. 1-1) core structures through a charge-mediated mechanism. Such binding is non-specific and can be inhibited by DNA and dextran sulphate (Mahdavi *et al.*, 2002).

1.2.5.5 Anti-Adhesion Treatment for *H. pylori* Infection

Adhesion is the initial step of bacteria contacting host cells. An increased understanding of the roles of adhesins in *H. pylori* infection has allowed the examination of an anti-adhesion strategy as a therapeutic alternative. Four oligosaccharides and five glycolipids were initially tested *in vitro* and 3'-sialyllactose (NeuAcα2,3Galβ1,4Glc) was found to be the best compound that could inhibit the *H. pylori* clinical isolates from attaching to the human gastrointestinal epithelial cells (Simon *et al.*, 1997). The anti-adhesive efficacy of 3'-sialyllactose was subsequently evaluated *in vivo* on eradication of *H. pylori* infection from rhesus macaques. 3'-sialyllactose decreased the colonization of *H. pylori* in only some of the infected animals. The addition of a proton pump inhibitor or bismuth subsalicylate failed to increase the cure rate (Mysore *et al.*, 1999). When 3'-sialyllactose was given to dyspeptic patients with *H. pylori* infection to test whether this oligosaccharide could suppress or clear *H. pylori* colonization in humans, the sugar was shown to be safe and well tolerated, but did not suppress or clear *H. pylori* colonization (Parente *et al.*, 2003). Due to the relatively low affinity of binding, cocktails of oligosaccharides and/or other anti-adhesive compounds, especially containing multimeric-binding sites, may increase the efficacy of an anti-adhesion approach. A recent study showed that mice fed with porcine milk containing Le^b and sialyl-Le^x displayed a lower degree of *H. pylori* colonization, compared to animals fed with porcine milk lacking the Le^b and sialyl-Le^x or fed with water only (Gustafsson *et al.*, 2005), confirming the potential of the anti-adhesion strategy in the prophylactic treatment.

1.2.6 Lipopolysaccharide and Lewis antigens

The lipopolysaccharide (LPS) of *H. pylori* is unique not only in its lipid A portion that generates very low endotoxic and immunobiological activities (Ogawa *et al.*, 1997; Ogawa *et al.*, 2003), but also in its O-antigens, which contain Lewis blood antigen structures (Aspinall and Monteiro, 1996; Aspinall *et al.*, 1996; Aspinall *et al.*, 1997; Chan *et al.*, 1995; Sherburne and Taylor, 1995). Such fucosylated oligosaccharides are also expressed on host gastric epithelium and deeper glands (Cordon-Cardo *et al.*, 1986; Kobayashi *et al.*, 1993; Taylor *et al.*, 1998). The structures of *H. pylori* LPS, synthesis of Lewis antigens, and how Lewis antigens contribute to *H. pylori* pathogenesis are described in this section.

1.2.6.1 Structures of *H. pylori* Lipopolysaccharides

The LPS, a main component of cell surface of *H. pylori*, shares a common architecture with other Gram-negative bacteria – lipid A, core oligosaccharide and O-antigen. Fresh clinical *H. pylori* isolates produce a high-molecular-weight smooth form of LPS (S-LPS), which is composed of the above-mentioned three components. In contrast, *H. pylori* strains that were subcultured numerous times on solid media, tend to lose their O side-chain, becoming a low-molecular-weight rough form (R-LPS) (Moran *et al.*, 1992). Strains bearing R-LPS can revert to S-LPS by growing in liquid media (Moran *et al.*, 1992).

LPS is attached to the outer membrane through its hydrophobic anchor lipid A. In Gram-negative bacteria, two 3-deoxy-*D-manno*-octulosonic acid (Kdo) sugars generally connect lipid A to the core oligosaccharide and O-antigen. The typical backbone of lipid A is a β1',6-linked disaccharide of glucosamine, which is phosphorylated and multiply acylated (Brozek and Raetz, 1990). The disaccharide backbone of lipid A in *E. coli* is polyacylated with (R)-3-hydroxymyristate at the 2-, 3-, 2'- and 3'- positions. The hydroxyl group of 2'- and 3'-linked fatty acyl chains are further esterified with laurate (C12) and myristate (C14), respectively (Brozek and Raetz, 1990). The lipid A of *H. pylori* is distinct from that of *E. coli* in that it lacks a 4'-phosphate group (Moran *et al.*, 1997; Suda *et al.*, 1997; Suda *et al.*, 2001) and only one, instead of two, Kdo sugars connect lipid A with the core oligosaccharide and O-antigen (Tran *et al.*, 2004). The triacyl or tetraacyl lipid A in *H. pylori* is predominant in both S-LPS and R-LPS with long acyl chains of 16 to 18 carbons in length, i.e. (R)-3-hydroxypalmitate (C16) or (R)-3-hydroxystearate (C18) (Moran *et al.*, 1997; Tran *et al.*, 2004). The minor species of lipid A of S-LPS is hexa-acylated (Moran *et al.*, 1997). The absence of an ester-bound 4'-phosphate and the presence of fatty acids with longer-chains in lipid A structure accounts for the very low endotoxic and immunobiological activities of *H. pylori* LPS, compared with that of other enterobacteria (Ogawa *et al.*, 1997; Ogawa *et al.*, 2003).

The O-antigen of *H. pylori* LPS has two unique features: incomplete fucosylation of the repeating Galβ1,4-*N*-acetylglucosamine (LacNAc) chain and the insertion of oligomers of *D*-glycero-*D*-manno-heptose (*DD*-Hep) between the O-antigen and core region (Aspinall *et al.*, 1997). The fucosylated poly-LacNAc chain contains Lewis blood antigen structures (Aspinall and Monteiro, 1996; Aspinall *et al.*, 1996; Aspinall *et al.*, 1997; Chan *et al.*, 1995; Sherburne and Taylor, 1995), which are also expressed on both gastric surface epithelium and deeper glands (Cordon-Cardo *et al.*, 1986; Kobayashi *et al.*, 1993; Taylor *et al.*, 1998). More than 80% of *H. pylori* strains express Type II Lewis antigens (Le^x and/or Le^y) and half of them express both (Aspinall and Monteiro, 1996;

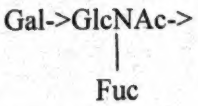
Monteiro *et al.*, 1998a; Sherburne and Taylor, 1995; Wirth *et al.*, 1996). A smaller proportion of *H. pylori* strains express Type I Lewis blood group antigens (Le^a and/or Le^b) (Monteiro *et al.*, 1998a) and a very small number of *H. pylori* strains express sialyl-Le^x (Monteiro *et al.*, 2000; Wirth *et al.*, 1996). A study shows that in 94 *H. pylori* isolates examined, twelve (13%) express Le^b, 3 (3%) express Le^a, and 2 (2%) express sialyl-Le^x (Wirth *et al.*, 1997). The O-antigen from some *H. pylori* strains shows no reaction to antibodies against Le^x, Le^y, Le^a, or Le^b so they are named non-typeable O-antigens (Rasko *et al.*, 2001). Comparison of Le^x and Le^y expression of *H. pylori* isolated from children and adults indicates that those from children express more Le^x but less Le^y than isolates from adults (Munoz *et al.*, 2001).

Six distinctive O-antigen serotypes have been identified in the LPS of *H. pylori*, designated serotype O1-O6 (Mills *et al.*, 1992) (Table 1-1). Serotype O1 LPS contains an elongated Lewis antigen chain connected to an elongated core polysaccharide. The O-antigen of strain NCTC11637 belongs to this group. It contains extended chains of fucosylated and nonfucosylated LacNAc units, terminated by a di- or trimeric Le^x determinant (Aspinall *et al.*, 1996). The O-antigen of strain P466 (isolated from a dyspeptic patient) also possesses similar extended chains of fucosylated and nonfucosylated LacNAc units containing multiple internal Le^x residues terminated by a Le^y determinant (Aspinall and Monteiro, 1996; Aspinall *et al.*, 1997). Serotype O2 LPS is composed of a core polysaccharide and a new extended O-antigen chain that is devoid of Lewis antigen structures but contains alternating 2- and 3-monosubstituted α-*D*-Glc residues. The O-antigen of strains UA861 and O2 shows such features. It is composed of an elongated LacNAc backbone, with approximately half of the GlcNAc units carrying a

Table 1-1. Schematic representation of *H. pylori* O-antigen structures with serotypes O1-O6. Adapted from (Aspinall *et al.*, 1997).

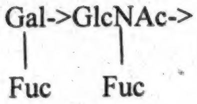
Serotype	Strains	Lewis antigen terminus	Extended O-antigens	Heptan	Core Oligosaccharides (OS)
O1	NCTC11637 (Le^x) P466 (Le^y)	Gal->GlcNAc-> | | ±Fuc Fuc	(Gal->GlcNAc)->(Gal->GlcNAc)->$_n$ | Fuc n_n		Core-OS
O2	UA861 O2	Gal->GlcNAc->	(Gal->GlcNAc)->(Gal-GlcNAc)->$_n$ | (Glc)$_n$ | (Glc)$_n$		Core-OS
O3	O3 (Le^x/Le^y)	Gal->GlcNAc-> | | ±Fuc Fuc	(Gal->GlcNAc)->(Gal->GlcNAc)->$_n$ | Fuc n_n	(-3DDHep-->)$_n$	Core-OS
O4	PJ2		(Glc->Glc->)$_n$	-3DDHep-->	Core-OS
O5	O5	Not yet characterized.	Not yet characterized.	Not yet characterized.	Core-OS
O6	MO19 (Le^y)	Gal->GlcNAc-> | | Fuc Fuc	-Gal->	(-3DDHep-->)$_n$	Core-OS

Possible Lewis antigen termini:



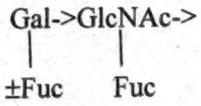
$$\text{Gal}\text{->}\underset{\substack{|\\ \text{Fuc}}}{\text{GlcNAc}}\text{->}$$

Le^x



$$\underset{\substack{|\\ \text{Fuc}}}{\text{Gal}}\text{->}\underset{\substack{|\\ \text{Fuc}}}{\text{GlcNAc}}\text{->}$$

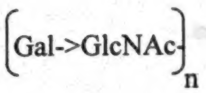
Le^y



$$\underset{\substack{|\\ \pm\text{Fuc}}}{\text{Gal}}\text{->}\underset{\substack{|\\ \text{Fuc}}}{\text{GlcNAc}}\text{->}$$

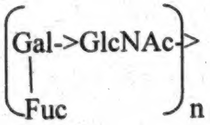
Le^x/Le^y

Possible extended oligomeric chain



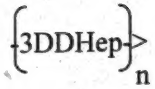
$$\left[\text{Gal->GlcNAc-}\right]_n$$

poly LacNAc chain



$$\left[\underset{\substack{|\\ \text{Fuc}}}{\text{Gal}}\text{->GlcNAc->}\right]_n$$

poly internal Le^x



$$\left\{\text{3DDHep->}\right\}_n$$

Heptan extended to core oligosaccharides
via 2- and 6-linked DD-Hep residues

terminal α-*D*-Glc residue at the O-6 position. The O-antigen chain is terminated by a LacNAc epitope but does not express a terminal Le^x or Le^y (Britton *et al.*, 2005; Monteiro *et al.*, 1998b). Serotype O3 LPS possesses lengthy Lewis antigen structures linked to the heptoglycan chain, such as that of *H. pylori* strain O3 (Aspinall *et al.*, 1997). Serotype O4 LPS expresses glucosylated Lewis antigens. The O-antigen from a clinical isolate strain PJ2 belongs to this group. It lacks the *DD*-heptoglycan and Lewis blood antigen but contains a core α1,6-glycan chain structure (Altman *et al.*, 2003). To my knowledge, the O-antigen structure of *H. pylori* serotype O5 has not yet been characterized. Serotype O6 LPS is composed of the heptoglycan domain either capped by a short Lewis antigen chain or short of Lewis antigen determinants. A good example is the O-antigen of strain MO19, which was isolated from asymptomatic patient. The O-antigen of this strain consists of a single Le^y epitope connected via a 3-linked β-D-Gal to the heptoglycan chain, which is then linked to the core oligosaccharides (Aspinall and Monteiro, 1996; Aspinall *et al.*, 1997; Monteiro *et al.*, 2000) (Table 1-1). The serotype O1-like LPS is the most common LPS type in *H. pylori* strains (Monteiro, 2001). On the other hand, some *H. pylori* strains express Type I Lewis blood antigens on their O-antigens. For instance strain UA948 carries both Le^x and Le^a structure; strain UA955 expresses a terminal α-L-Fuc-1,4-β-D-GlcNAc structure while strain J233 carries Type I, H-Type I (Fig. 1-1) and an elongated LacNAc chain (Monteiro *et al.*, 1998a).

1.2.6.2 Synthesis of Lewis Antigens

The synthesis of Lewis antigen determinants requires a series of glycosyltransferases (section 1.3) with fucosylation being the final step. The genes for *H. pylori* LPS synthesis are not clustered as seen in *E. coli*, *Salmonella typhimurium* and *Yersinia enterocolitica*

(Heinrichs *et al.*, 1998; Kaniuk *et al.*, 2002), but are scattered throughout the genome, except for the genes encoding proteins responsible for GDP-Fuc synthesis, *rfbM*, *rfbD* and *wbcJ*, which are clustered (McGowan *et al.*, 1998).

Lewis antigen production is catalyzed by α1,2 and α1,3/4 fucosyltransferases (FucTs) (section 1.4.4). α1,3/4 FucTs transfer fucose from donor GDP-fucose (GDP-Fuc) to the subterminal GlcNAc moiety of Type II or Type I acceptor with an α1,3 or α1,4 linkage (Fig. 1-2). α1,2 FucTs transfer fucose from GDP-Fuc to the terminal galactose moiety of Type II or Type I acceptor with an α1,2 linkage (Fig. 1-2). Difucosylated Lewis antigens (Le^y and Le^b) can be synthesized via two pathways: terminal fucosylation (α1,2 linkage) followed by subterminal fucosylation (α1,3 or α1,4 linkage), or subterminal fucosylation followed by terminal fucosylation (Wang *et al.*, 2000). It has been reported that the former pathway is primarily utilized in mammalian cells, whereas, the latter pathway is predominantly used in *H. pylori* (Wang *et al.*, 2000) (Fig. 1-3).

1.2.6.3 Roles of Lewis Antigens in Pathogenesis

1.2.6.3.1 Molecular Mimicry and Autoimmunity

As both *H. pylori* cell surface and gastric tissue express similar Lewis antigen structures, molecular mimicry between Lewis structures on *H. pylori* LPS and gastric tissues has been proposed to be a possible mechanism that *H. pylori* uses to evade the immune response to maintain a long-term infection (Appelmelk *et al.*, 1997). It was also suggested that *H. pylori* Lewis antigens induce production of antibodies and cause anti-gastric autoreactivity leading to tissue damage (Heneghan *et al.*, 2001; Wirth *et al.*, 1997). Some evidence suggest that Lewis antigens are not the antigenic components of LPS (Faller *et al.*, 1998; Yokota *et al.*, 1998), but rather that the antigen could be a

Fig. 1-2. The synthesis of Lewis X and Lewis A from Type II and Type I substrates by α1,3 and α1,4 fucosyltransferases (FucTs), respectively. Lewis X and Lewis A can be further fucosylated by α1,2 FucT to produce the tetrasacchardies Lewis Y and Lewis B (shown in green).

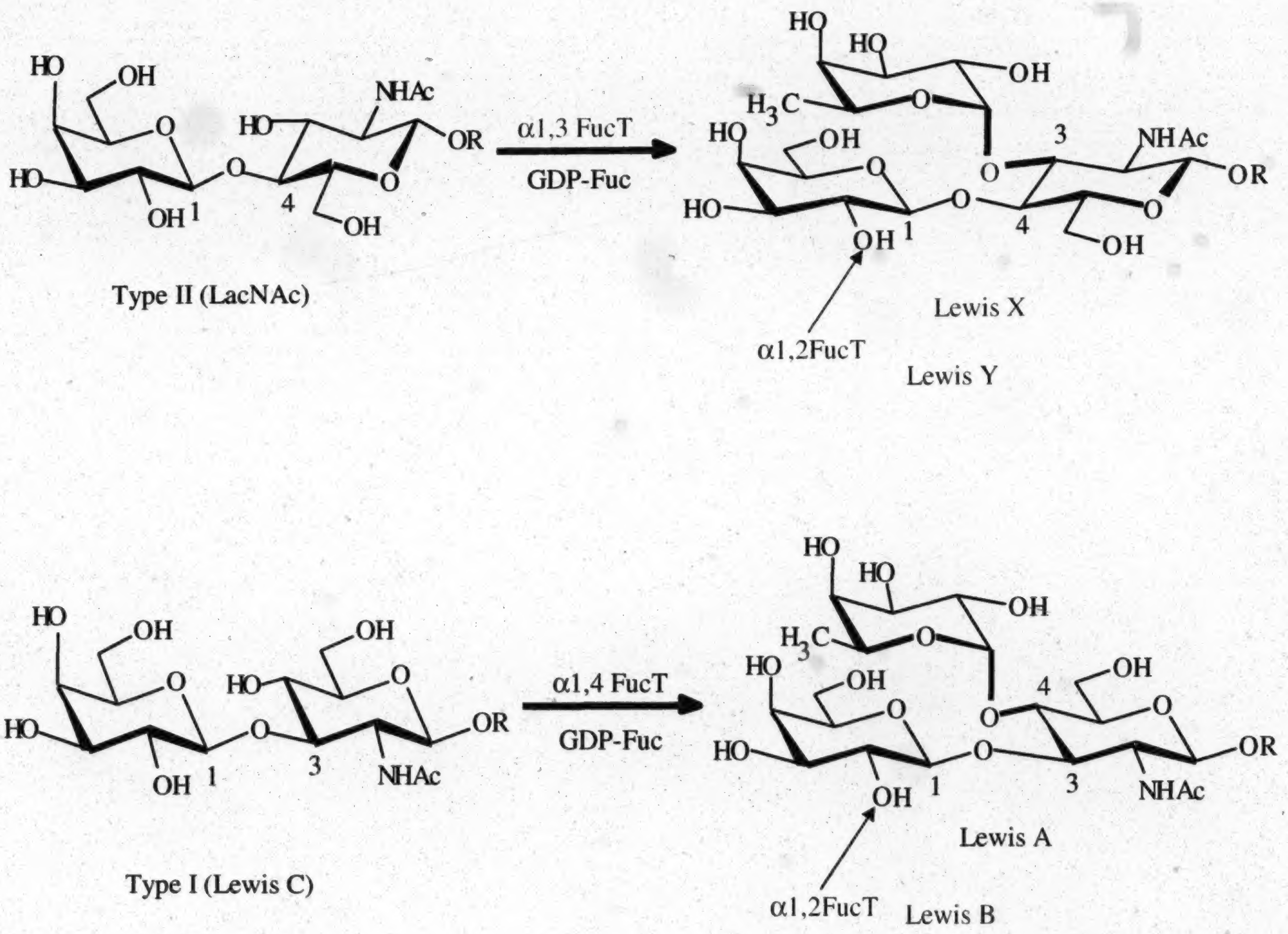
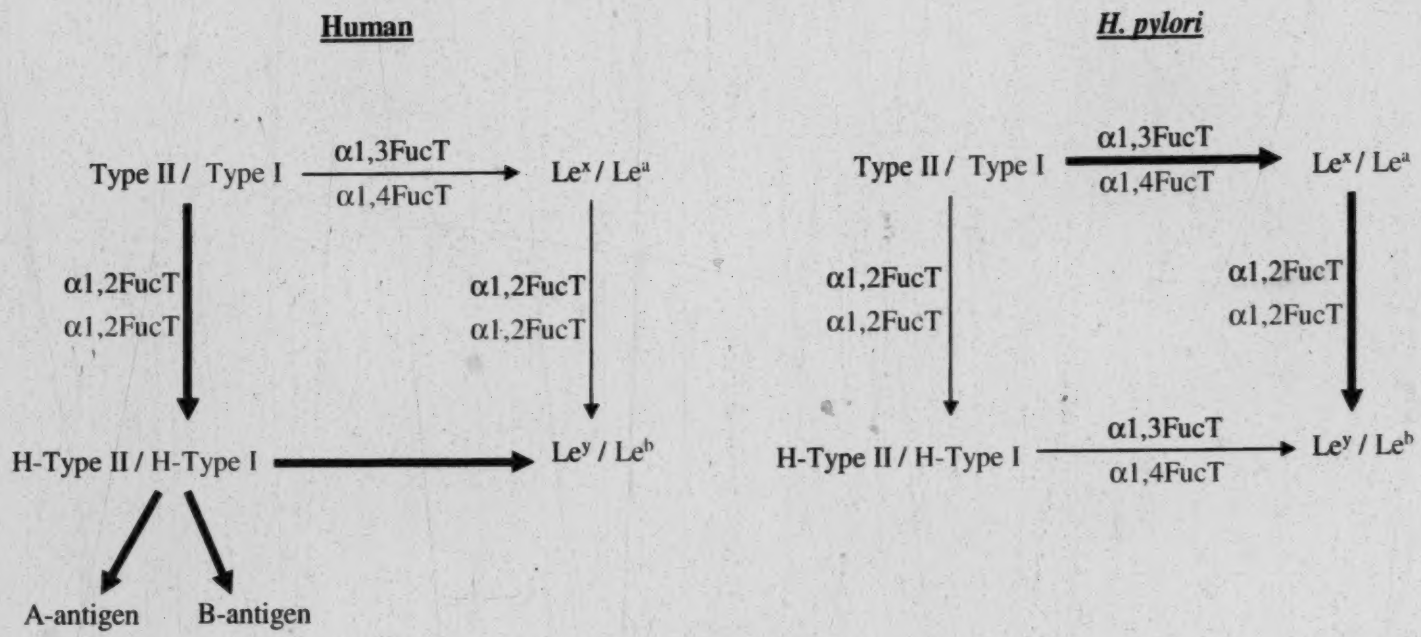

Type II (LacNAc)
α1,3 FucT
GDP-Fuc
Lewis X
α1,2FucT
Lewis Y
Type I (Lewis C)
α1,4 FucT
GDP-Fuc
Lewis A
α1,2FucT
Lewis B

Fig. 1-3.Comparison of the synthesis pathways of Lewis antigens by α1,3/4 and α1,2 fucosyltransferases (FucTs) in humans and in *H. pylori*. The black and blue color denotes the Type II and Type I series Lewis antigen production, respectively. The thickness of the arrows indicates the relative level of enzyme activity. It was proposed that the difucosylated Lewis antigens (Le^y and Le^b) are synthesized predominantly *via* the terminal fucosylation followed the subterminal fucosylation in human (left), whereas in *H. pylori* it is primarily produced *via* the subterminal fucosylation followed by the terminal fucosylation (right) (Wang *et al.*, 2000). Lewis X: Le^x, Lewis Y: Le^y, Lewis A: Le^a, Lewis B: Le^b. The structures of Lewis blood antigens are shown in Fig.1-2.


Human
Type II / Type I
α1,3FucT
α1,4FucT
Le^x / Le^a
α1,2FucT
α1,2FucT
α1,2FucT
α1,2FucT
H-Type II / H-Type I
Le^y / Le^b
A-antigen
B-antigen
H. pylori
Type II / Type I
α1,3FucT
α1,4FucT
Le^x / Le^a
α1,2FucT
α1,2FucT
α1,2FucT
α1,2FucT
H-Type II / H-Type I
α1,3FucT
α1,4FucT
Le^y / Le^b

structure in the polysaccharide chain of LPS, which has yet to be defined (Yokota *et al.*, 1998).

1.2.6.3.2 Adhesion, Colonization and Association with Severe Pathology

To study the pathogenesis of *H. pylori* infection, animal models have been developed to characterize processes of bacterial adhesion and colonization, as well as development of gastritis, ulcer and carcinoma. Mice, rhesus macaques, Mongolian gerbil, and ferrets are commonly used, and each animal model has advantages and disadvantages. For more details the reader is directed to several comprehensive reviews (Eaton, 1999; Lee, 1998; O'Rourke and Lee, 2003; Tatematsu *et al.*, 2003). Due to their availability and low cost, the majority of the studies have been undertaken in mice. It is worth noting that murine epithelial cells do not normally express Le^b (the ligand of BabA), thus any colonization data obtained from mouse models must be cautiously interpreted when extrapolating their results to human infection (Guruge *et al.*, 1998)

As described above, the Lewis antigens of *H. pylori* play a minor role in bacterial adhesion (section 1.2.5.3). There has been some evidence to suggest that such fucosylated oligosaccharides are also involved in *H. pylori* colonization. *H. pylori* strains expressing R-LPS or deficient in $Le^{x/y}$ fail to colonize the mouse stomach, in contrast to the successful colonization by strains expressing S-LPS or $Le^{x/y}$ positive antigens (Martin *et al.*, 2000; Moran *et al.*, 2000). Nevertheless, other studies show that Le^x and/or Le^y negative mutants colonize the mouse stomach equally well as strains expressing Le^x and Le^y (Suresh *et al.*, 2000; Takata *et al.*, 2002). A *H. pylori* strain PJ2, devoid of the DD-heptoglycan and Lewis antigens but containing the core α1,6-glycan chain, colonize the mouse stomach equally to DD-heptoglycan- and Lewis antigen-producing strains

(Altman *et al.*, 2003). This implies that neither Lewis blood antigens nor the extended DD-heptoglycan is a prerequisite for colonization in the mouse model.

To mimic the *H. pylori* infection that occurs in humans and primates, transgenic mice were created that express Le^b in their gastric pits. Transgenic mice and their nontransgenic littermates were inoculated with 11 $CagA^+$ and $VacA^+$ clinical isolates (Guruge *et al.*, 1998). No difference was observed in the number of viable *H. pylori* recovered from the stomachs of two groups of mice 8 or 16 weeks after inoculation, implying a similar colonization rate is established. However, bacteria in the transgenic mice expressing Le^b when compared with nontransgenic mice lacking Le^b expression show sustainable differences in distribution. *H. pylori* in the transgenic mice are associated with the mucous layer, gastric pit and the surface mucous cells wheras in non-transgenic mice the distribution of *H. pylori* is limited to the mucous layer only. Moreover, inflammation is more severe and the production of autoantibodies to Le^x by parietal cells were detected more often in the transgenic versus nontransgenic mice, and the transgenic mice showed a higher chance of developing MALT lymophoma after 16 weeks (Guruge *et al.*, 1998). Therefore, it appears that bacterial attachment affects host immune responses. A *H. pylori* strain that confers BabA-Le^b binding is more likely to induce severe gastric pathology than a strain that is unable to form tight adhesion with gastric tissue, even though both strains display similar colonization rates.

Even though Lewis antigens only play a minor role in *H. pylori* adhesion and colonization, it seems that the expression of such fucosylated oligosaccharides is very often correlated with development of clinical symptoms. For instance, *H. pylori* strains isolated from asymptomatic individuals, display a decreased level of Le^x expression, an absence of Le^a and/or Le^b structures, an increase in nontypeable Lewis blood antigens on the LPS (Rasko *et al.*, 2001), or express an extended heptoglycan chain lacking Lewis antigen determinants (Monteiro *et al.*, 2001), as compared to symptomatic patients. Another study demonstrated that, even though the Lewis antigen-negative *H. pylori* strains colonize the stomach of C57BL/6J mice equally well as the strains with intact Lewis antigens, only strains with intact Lewis antigen cause extensive gastritis and gastric damage (Eaton *et al.*, 2004).

1.2.6.3.3 Internalization

H. pylori are predominantly localized within the mucosal layer (Schreiber *et al.*, 2004), and very few are internalized into gastric cells *in vivo* (Bode *et al.*, 1987; Kazi *et al.*, 1990). However, *H. pylori* strains expressing Le^x were found to be internalized highly by both human cultured gastric adenocarcinoma (AGS) cell lines and the epithelium of human gastric xenografts in nude mice. In contrast, isogenic mutants that do not express Le^x display a very low level or no internalization (Lozniewski *et al.*, 2003). It is not yet known how Le^x enhances bacteria internalization, and how significant the intrinsic low level of *H. pylori* internalization is to pathogenesis.

1.2.6.3.4 Modulation of Host Immune Response via C-type lectin DC-SIGN

Recently, *H. pylori* Lewis antigens were identified as a ligand for dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (Appelmelk *et al.*, 2003; Bergman *et al.*, 2004). Dendritic cells are found in stomach lamina propria or close to the gastric lumen during *H. pylori* infection (Bergman *et al.*, 2004). The specific Lewis blood antigen structures, including Le^x (monomeric up to [Le^x]^4, but not the polymeric Le^x), Le^y, Le^a, Le^b and H-Type II (but not H-Type I) structures, are the ligands of DC-SIGN.

Disaccharides, Fucα1,4GlcNAc and Fucα1,2Galβ, can also bind to DC-SIGN but with lower affinity (Bergman *et al.*, 2004).

Dendritic cells are professional antigen presenting cells playing a significant role in inducing the innate and adaptive immune response. It was reported that *H. pylori* activates dendritic cells and promotes their maturation in a *cag* PAI- and VacA-independent manner (Kranzer *et al.*, 2005). Activated dendritic cells secrete interleukin-6 (IL-6), IL-8, IL-10, IL-12, IL-1β and tumor necrosis factor (TNF) (Kranzer *et al.*, 2005). Among these interleukins, dendritic cells preferentially produce IL-12, and lesser amounts of IL-6 and IL-10 (Guiney *et al.*, 2003). As a Th1 cytokine, IL-12 promotes natural killer (NK) activation and induces Th1 cell response (Hafsi *et al.*, 2004).

It has been shown that acute *H. pylori* infection features in a predominantly Th1 cell response – high production of interferon-γ, TNF-α and IL-12 (D'Elios *et al.*, 2005; Lohoff *et al.*, 2000; Sommer *et al.*, 1998; Wen *et al.*, 2004) and development of severe gastric inflammation (i.e. peptic ulcers). This was manifested in both clinical patients (Bamford *et al.*, 1998; D'Elios *et al.*, 1997b) and rhesus macaques (Mattapallil *et al.*, 2000). A very small number of infected patients, whose host responses were apparently skewed to the abnormal induction of B-cell growth and uncontrolled proliferation, develop neoplastic transformation and gastric MALT lymphoma (D'Elios *et al.*, 2005). Nevertheless, most of the individuals who are infected with *H. pylori* but display either uncomplicated gastritis or no clinical symptoms are of Th0 cell phenotype, secreting both Th1 and Th2 cytokines (D'Elios *et al.*, 1997a; D'Elios *et al.*, 2005). It was reported that Th2 cytokines such as IL-4 and IL-10 are able to balance and quench some of the detrimental effects of a polarized Th1 response (Bodger *et al.*, 1997; Smythies *et al.*, 2000).

It was shown that the binding of the *H. pylori* Lewis antigens to the DC-SIGN ligand on the dendritic cells enhanced the production of IL-10, which is able to promote the Th2 while blocking the Th1 cell response. As such, a polarized Th1 effect can be turned into a mixed Th1-Th2 cell response through Lewis antigen-DC-SIGN interaction so that an acute infection is switched into a chronic infection (Bergman *et al.*, 2004). The expression of *H. pylori* Lewis blood group antigens is regulated by phase variation (section 1.4.4.4). Hence, Lewis antigens through phase variation aid *H. pylori* in modulating the host immune response thereby promoting persistent colonization (Bergman *et al.*, 2004).

1.2.6.4. Acidic Conditions Induce Modification of LPS Expression

LPS expression is dependent on the pH of the environments. Examination of the LPS expression of *H. pylori* strain 26695 by Moran *et al.* shows that at pH 5, the fucosylation at internal sites of the LacNAc chain was decreased so less internal Le^x was formed. Instead, the majority of the non-fucosylated GlcNAc becomes galactosylated at the O-6 position. In addition, the Le^x structure expressed at the terminus of the LacNAc chain at pH7 is further fucosylated to generate a Le^y structure at pH 5 (Moran *et al.*, 2002). How such changes might benefit the bacteria in infection was not investigated in this study. Nevertheless, another study shows that in strain 60190, acidic conditions induce expression of w*bcJ,* the gene predicted to encode a protein responsible for the conversion of GDP-mannose to GDP-Fuc. The isogenic *wbcJ* null mutant strain (devoid of Le^x and Le^y expression) is more sensitive to acid stress (pH 3.5) than wild type (McGowan *et al.*, 1998). It appears that *H. pylori* is able to sense the pH change and to alter the expression of Lewis antigen, by modifying the fucose availability and the FucT activity levels, thereby enhancing bacterial survival during acid stress. Therefore, it remains to be examined if decreased pH regulates the expression of Lewis antigens in only some or all strains, and if the Lewis antigen modification follows a specific pattern (i.e. less internal Le^x and expression of terminal Le^y).

1.3 Glycosyltransferases

1.3.1 Glycosyltransfereases and Glycosylation

Glycosyltransferases (GTs) (EC 2.4.1.x.y) transfer sugars to various targeted acceptors, forming glycosidic bonds. The donor sugars are commonly hexoses with either a D- or L-configuration, and the majority of them are sugar nucleotide mono- or diphosphates (NMP-sugar or NDP-sugar). Acceptor molecules show great diversity, including oligosaccharides, proteins, nucleic acids, lipids and biologically active natural products that contain sugar moieties (i.e. erythromycin A and vancomycin *etc*) (Hu and Walker, 2002). Campbell *et al.* have divided the GT superfamily into subfamilies according to the identity of the donor and acceptor sugar, as well as sequence homology (Campbell *et al.*, 1997). Currently more than 14,000 known or putative GTs are listed in GT family of the Carbohydrate-Active enZYme (CAZY) database (http://afmb.cnrs-mrs.fr/CAZY/) and 78 subfamilies have been defined, with α1,3/4 FucT belonging to superfamily GT10.

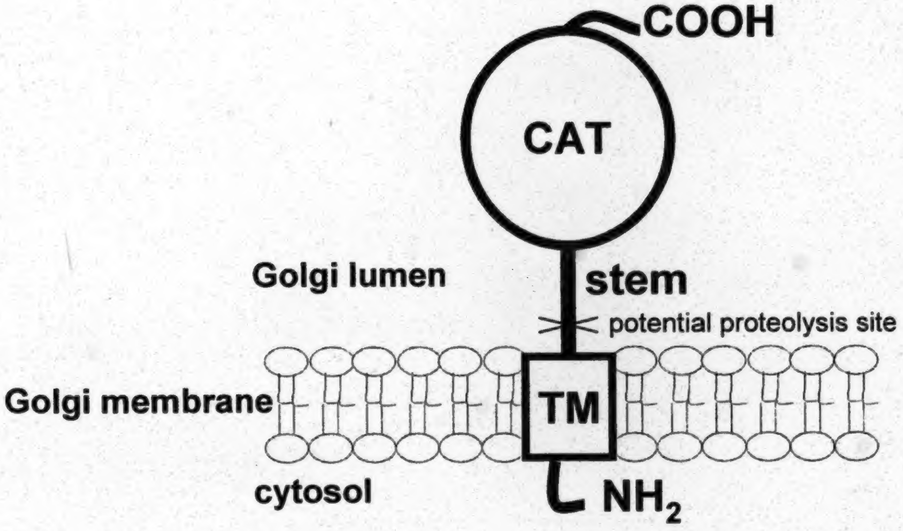
Glycosylation reactions may proceed with either an inversion or retention of stereochemistry of the C-1 position in the donor sugar. The direct displacement mechanism of the inverting reaction is straightforward. A residue in the GT acts as a general base that extract a proton from the reactive hydroxyl group of the acceptor, aiding the acceptor hydroxyl in acting as a catalytic nucleophile to attack the C-1 of the donor, which leads to the inversion of stereochemistry (also called S_N2 attack) (Unligil and Rini, 2000). The mechanism of the retaining reaction is believed to follow a two-step double displacement scheme via a covalent glycosyl-enzyme intermediate. Firstly a residue in the GT acts as a nucleophile attacking the anomeric center (C1) of the donor sugar leading to formation of a glycosyl-enzyme intermediate. A metal ion or a catalytic acidic residue then assists the cleavage of the C1-O1 bond (first displacement). Subsequently, a general base of the GT deprotonates the reactive hydroxyl of the acceptor sugar, therefore enable it to attack the anomeric center of the enzyme-linked donor (second displacement). The retaining reaction, therefore, generally requires two catalytic residues from the GT, and a glycosyl-enzyme intermediate is formed. Due to the difficulty in trapping the glycosyl-enzyme intermediate experimentally, though this mechanism was originally proposed in 1990 (Sinnott, 1990), it is still controversial (Davies, 2001; Withers *et al.*, 2002). An alternative mechanism for the retaining reaction was suggested, originally for α1,4 galactosyltransferase LgtC from *Neisseria meningitides*. This mechanism proceeds with a single step: the reactive hydroxyl group of the acceptor sugar directly attacks the anomeric C-1 of the donor sugar from the same side that the nucleotide portion of the donor departs (also called S_Ni attack) (Ly *et al.*, 2002; Persson *et al.*, 2001). Besides LgtC, bovine α1,3 galactosyltransferase (Boix *et al.*, 2002), trehalose-6-phosphate synthase OtsA (Gibson *et al.*, 2002), α1,4 N-acetylhexosaminyltransferase Extl2 (Lobsanov *et al.*, 2004), mannosylglycerate synthase MGS (Flint *et al.*, 2005) have been suggested to follow this mechanism, but again this has not been verified experimentally. Interestingly, computational modeling predicts that LgtC follows a very similar mechanism to the S_Ni attack described above (Tvaroska, 2004). However, the same research group who proposed the S_Ni attack mechanism for LgtC in 2001 successfully trapped the glycosyl-enzyme intermediate with a mutated LgtC (Gln^{189}→Glu) (Lairson *et al.*, 2004). Their HPLC/ESMS analysis data showed that it was not the Glu^{189}, but its neighboring residue Asp^{190} that is covalently bound to the galactosyl moiety, suggesting that Asp^{190} might act as a catalytic base. However, the crystal structure of the mutant LgtC showed that Asp^{190} is not in an appropriate position and orientation with respect to the donor sugar. Therefore if it functions as a catalytic base, a conformational change would need to occur during the catalysis (Lairson *et al.*, 2004). This opens the possibility that LgtC might still follow the double displacement scheme for catalysis. The mechanistic basis for retaining reaction remains an unanswered question.

1.3.2 Domain Construction

In eukaryotic cells, GTs are found to be localized within the Golgi apparatus, endoplasmic reticulum (ER), cytoplasm or nucleus. Notably, a recent study showed that most of the enzymes belonging to the GT-A family (see section 1.3.3) are localized in the lumen of Golgi, whereas GT-C fold (see section 1.3.3) enzymes are localized in the lumen of ER. The enzymes belonging to the GT-B family (see section 1.3.3) are found in the four locations listed above (Kikuchi *et al.*, 2003). The GTs residing within the Golgi apparatus have been extensively studied. These enzymes are type II transmembrane proteins with common domain architecture: an N-terminal cytoplasmic tail, a transmembrane domain, and an extended stem region followed by a large globular C-terminal catalytic domain (Fig. 1-4). The soluble forms of the enzymes present in biological fluids are produced by proteolysis at the luminal stem region (D'Agostaro *et al.*, 1989). The length of the stem region in different GTs is variable. The longest stem region reported to date is approximately 400 amino acids in UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase (Ten Hagen *et al.*, 1998), whereas the shortest stalk domain appears to be only a few amino acids if there is one at all, in β-galactoside-α2,6 sialyltransferase III (Sjoberg *et al.*, 1996). The flexible stem region is believed to play a role in positioning the catalytic domain away from the lipid layer where the transmembrane domain attaches, so that the catalytic domain can access substrate. The stem region can be defined as a fragment that is located immediately downstream of the transmembrane domain and enzyme activity is retained when it is deleted (Breton *et al.*, 2001). Nevertheless, there is at least one exception to this generalization: the stem region (about 50 amino acids) of human β-galactoside α2,6 sialyltransferase seems to participate in the recognition of acceptor, possibly by exerting steric control on the catalytic domain allowing it to distinguish different acceptor structures (Legaigneur *et al.*, 2001).

Currently there are two mechanisms that account for Golgi retention of GTs: kin recognition (Nilsson *et al.*, 1993; Nilsson *et al.*, 1996) and the lipid layer model (Munro, 1995). In the kin recognition model, GTs form aggregates by oligomerization so that the enzyme is retained in the Golgi apparatus (Nilsson *et al.*, 1993; Nilsson *et al.*, 1996). In the lipid layer model, the hydrophobic transmembrane domain is believed to retain the enzymes in the Golgi apparatus and the length of transmembrane domain specifies the particular localization (Munro, 1995). Nevertheless, neither model fully explains GT localization. A study examining the expression in Chinese hamster ovary cells of six

Fig 1-4. Schematic of typical domain construction of Type II transmembrane glycosyltransferases located in the Golgi apparatus. NH_2: N-terminus; COOH: C-terminus, TM: transmembrane domain; CAT: catalytic domain.


COOH
CAT
Golgi lumen
stem
potential proteolysis site
Golgi membrane
TM
cytosol
NH$_2$

recombinant GTs, two N-acetylglucosaminyltransferases (core 1 β1,3-N-acetylglucosaminyltransfease [C1-β3GnT] and core 2 β1,3-N-acetylglucosaminyltransfease [C2GnT-I]), an α1,2 FucT (FucT I), an α1,3 FucT (FucT VII) and two sialyltransferases (α2,3 sialyltransferase I [ST3Gal-I] and α2,6 sialyltransferase I [ST6Gal-I]), shows that all six GTs except FucT VII are secreted into the medium, but all of them except ST3Gal-I are present within cells in a combination of monomers and dimers. The secreted C2GnT-I and ST6-Gal-I are exclusively monomers; whereas the secreted FucT I are dimers and the secreted C1-β3GnT are a mixture of monomers and dimers. Therefore, dimerization does not always lead to Golgi retention (El-Battari *et al.*, 2003). Another example is the α1,2 FucT, FucT I, in which the cytoplasmic tail (8 amino acids in length) determines Golgi enzyme localization (Osman *et al.*, 1996). Specifically, the hydroxyl group of the serine residue at position 5 is essential (Milland *et al.*, 2001). Indeed, the transmembrane domain and its flanking sequences, including the cytosolic tail and the luminal stem region, can all contribute to the localization of different GT members (Breton *et al.*, 2001).

1.3.3 Structural Folds

GTs display highly divergent protein sequences and utilize a vast variety of distinctive acceptor molecules to produce an enormous diversity of products. It therefore might be expected that GTs would exhibit numerous structural folds as the glycosidase superfamily (EC 2.4.1.x.y) (Bourne and Henrissat, 2001; Henrissat and Davies, 1997). Surprisingly, GTs adopt a very limited number of folds, of which GT-A and GT-B folds are well understood. To date there are twenty-four GTs with resolved crystal structures, and all of them belong to either the GT-A or GT-B fold family (Bourne and Henrissat, 2001; Unligil and Rini, 2000) (Table 1-2). It is worth mentioning that only the catalytic domain of these GTs have been crystallized after a deletion of either the N-terminal transmembrane domain (Boix *et al.*, 2002; Gastinel *et al.*, 1999; Gastinel *et al.*, 2001; Patenaude *et al.*, 2002; Ramakrishnan and Qasba, 2002; Unligil *et al.*, 2000) or C-terminal membrane associated fragment (Chiu *et al.*, 2004; Persson *et al.*, 2001). It appears that deletion of these membrane domains or fragments facilitates crystallization. Due to almost no sequence identity shared by different families of GT enzymes, methods to recognize the remote protein sequence homology including the three-dimensional position specific scoring matrix (3D-PSSM), iterative searches of sequence databases (PSI-Blast) and hidden Markov models have recently been developed. These methods are able to extract functional motifs, analyze and compare the secondary structures and analyze completely sequenced genomes to track the origins of protein-folds. Using these methods, a third GT fold, GT-C, has been identified (Kelley *et al.*, 2000; Kikuchi *et al.*, 2003; Liu and Mushegian, 2003). Nevertheless, GT-A, GT-B and GT-C do not seem to cover all of the folds employed by GTs. GT-D (or unclassified fold) is proposed as the fourth fold of the GT superfamily (Liu and Mushegian, 2003).

1.3.3.1 GT-A Fold

The GT-A fold has a common spatial structure, a N-terminal Rossmann-like domain with alternate extended β-strands and α-helices and a C-terminal domain composed mainly of β-strands. Seven β-strands within the Rossmann-like domain form a central sheet and are generally arranged with the 3214657 topology with strand 6 antiparallel to the remaining six β strands. The α-helices fill in two layers on each side of the plane (Liu and Mushegian, 2003). The N-terminal domain is involved in donor binding and the C-

Table 1-2. Glycosyltransferases with resolved crystal structures. Structural data are available at the 3D-glycosyltransferase database (http://www.cermav.cnrs.fr/glyco3d/). Only reference to the original work is shown. Adapted from (Breton *et al.*, 2005; Qasba *et al.*, 2005).

Organism	Glycosyltransferase	Name	GT family	Mechanism	GT-fold	Metal requirement	DXD motif	Hypervariable loop	Reference
Virus									
phage T4	β-Glucosyltransferase	BGT	GT63	Inverting	B	-	-	-	(Vrielink *et al.*, 1994)
phage T4	α-Glucosyltransferase	AGT	GT72	Retaining	B	-	-	-	(Lariviere *et al.*, 2005)
Prokaryotes									
Agrobacterium tumefaciens	Glycogen synthase I	AtGS	GT5	Retaining	B	-	-	-	(Buschiazzo *et al.*, 2004)
Amycolatopsis orietalis	β-epi-vancosaminyltransferase	GtfA	GT1	Inverting	B	-	-	-	(Mulichak *et al.*, 2003)
	β-Glucosyltransferase	GtfB	GT1	Inverting	B	-	-	-	(Mulichak *et al.*, 2001)
	β-vancosaminyltransferase	GtfD	GT1	Inverting	B	-	-	-	(Mulichak *et al.*, 2004)
Bacillus Subtilis	Putative glycosyltransferse	SpsA	GT2	Inverting	A	metal	DXD	134-136, 218-231	(Charnock and Davies, 1999)
Campylobacter jejuni	α2,3/2,8-sialyltransferase	CstII	GT42	Inverting	A	-	-	155-188	(Chiu *et al.*, 2004)
Escherichia coli	β1,4-N-Acetylglucosaminyltransferase	MurG	GT28	Inverting	B	-	-	-	(Ha *et al.*, 2000)
	Thehalose-6-phosphate synthase	OtsA	GT20	Retaining	B	-	-	-	(Gibson *et al.*, 2002)
	Heptosyltransferase II	RfaF	GT9	Inverting	B	-	-	-	PDB 1PSW
Neisseria meningitidis'	α1,4-Galactosyltransferase	LgtC	GT8	Retaining	A	-	-	75-80, 246-251	(Persson *et al.*, 2001)
Rhodothermus marinus	Mannosylglycerate synthase	MGS	GT78	Retaining	A	metal	DXD	-	(Flint *et al.*, 2005)
Eukaryotes									
yeast	α1,2-mannosyltransferase	Krep/Mnt1p	GT15	Retaining	A	metal	EXD	-	(Lobsanov *et al.*, 2004)
mouse	α1,4-N-acetylhexosaminyltransferase	Extl2	GT64	Retaining	A	metal	DXD	277-281	(Pedersen *et al.*, 2003)
	Polypeptide-α-N-acetylgalactosyltransferase	PpGalNAc-T1	GT27	Retaining	A	metal	DXH	-	(Fritz *et al.*, 2004)
rabbit	α-glucosyltransferase	Glycogenin	GT8	Retaining	A	metal	DXD	-	(Gibbons *et al.*, 2002)
	β1,2-N-Acetylglucosaminyltransferse I	GnT I	GT13	Inverting	A	metal	EXD	318-330	(Unligil *et al.*, 2000)
bovine	α1,3-Galactosyltransferase	α3GalT1	GT6	Retaining	A	metal	DXD	358-368	(Gastinel *et al.*, 2001)
	β1,4-Galactosyltransferase	β4GalT1	GT7	Inverting	A	metal	DXD	313-316, 345-365	(Gastinel *et al.*, 1999)
human	β1,3-Glucuronyltransferase	GlcAT-I	GT43	Inverting	A	metal	DXD	140-152	(Pedersen *et al.*, 2000)
	β1,3-Glucuronyltransferase	GlcAT-P	GT43	Inverting	A	metal	DXD	150-162	(Kakuda *et al.*, 2004)
	α1,3-N-Acetylgalactosyltransferase A	GTA	GT6	Retaining	A	metal	DXD	179-194	(Patenaude *et al.*, 2002)
	α1,3-Galactosyltransferase B	GTB	GT6	Retaining	A	metal	DXD	179-194	(Patenaude *et al.*, 2002)

terminus provides for acceptor recognition. Interestingly, Liu and Mushegian revealed that many GT-A fold enzymes also contain additional small β-sheets or β-hairpins that are located with an angle to the main β-sheets of the core of Rossmann-like domain. These β-sheets (or hairpins) are close to the catalytic residues of the central β-sheets and contribute to substrate binding and catalysis. (Liu and Mushegian, 2003).

Another feature of the GT-A fold is that the majority of enzymes in this family need divalent metal ions for catalysis as cofactors. These enzymes possess a functional DXD (or EXD) tripeptide motif with one or both acidic residues binding to the divalent ion, most commonly Mn^{2+} or Mg^{2+} (Hu and Walker, 2002; Qasba *et al.*, 2005). The divalent metal ion generally binds to the enzyme first through one or two of the acidic residues within the DXD (EXD) motif. Water molecules also interact with the ion to form an octahedral coordination. The donor substrate subsequently binds to the metal-bound-enzyme with the two negatively-charged oxygen atoms from the α-phosphate and β-phosphate of nucleotide-sugar donor replacing the water molecules. As such, the metal ion aids in donor binding, stabilizes the transition state and accelerates the departure of the nucleotide portion of sugar (Hu and Walker, 2002; Qasba *et al.*, 2005). At least two enzymes that belong to the GT-A fold family, α1,4 galactosyltransferase LgtC (CAZY family 8) (Persson *et al.*, 2001) from *Neisseria meningitides* and the sialyltransferase (CstII) from *Campylobacter jejuni* (CAZY family 42), lack a DXD (or EXD) motif (Chiu *et al.*, 2004) (Table 1-2). The fold of CstII is unique in that the seven-stranded β-sheets with the α/β/α sandwich structure are all parallel and display the 8712456 topology (Breton *et al.*, 2005; Chiu *et al.*, 2004). So it has also been grouped into the unclassified fold subfamily, together with CAZY family 29 and 73 (Breton *et al.*, 2005; Kikuchi *et al.*, 2003; Liu and Mushegian, 2003). With increased characterization of the unknown GT folds, it will become clearer into which type of fold family CstII should be categorized.

For the members of GT-A family catalyzing inverting reactions, it is relatively easy to identify the structurally equivalent carboxylate, generally Asp or Glu, acting as the catalytic base to activate the hydroxyl group of the acceptor sugar. Examples include Asp^{191} in *Bacillus subtilis* glycosyltransferase SpsA (CAZY family 2) (Tarbouriech *et al.*, 2001), Asp^{318} in bovine β1,4 galactosyltransferase (CAZY family 7), Asp^{291} in rabbit N-acetylglucosaminyltransferase (CAZY family 13) (Unligil *et al.*, 2000), Glu^{281} in human glucuronyltransferase I (CAZY family 43) (Pedersen *et al.*, 2000) and possibly Asp^{332} in UDP-glucosyltransferase GtfB (Mulichak *et al.*, 2001). In contrast, the catalytic base can not be easily identified in retaining enzymes (Lairson *et al.*, 2004; Persson *et al.*, 2001).

1.3.3.2 GT-B Fold

Proteins in the GT-B family contain two dissimilar Rossmann-folds (α/β/α structure) separated by a deep cleft where the donor and acceptor bind. In contrast to the GT-A family, the C-terminal subdomain that is rich in glycines and prolines recognizes the nucleotide-sugar donor and the N-terminal region provides the acceptor binding site (Hu and Walker, 2002; Hu *et al.*, 2003; Mulichak *et al.*, 2001). GT-B fold enzymes do not have a DXD or EXD motif (Hu and Walker, 2002; Qasba *et al.*, 2005), and they do not require metal ions for enzyme catalysis, though in some cases the ion accelerates the reaction rate. It is worth noting that the DXD (EXD) motif is widely present not only in GTs (71%) but also in the glycosidase superfamily (69%) (Coutinho *et al.*, 2003). Therefore, the occurrence of the DXD (EXD) motif alone in a GT enzyme cannot be used as diagnostic criteria to distinguish whether an enzyme is likely to possess either the GT-A or the GT-B fold.

GT-A and GT-B enzymes have different active sites and distinctive catalytic mechanisms, yet they share some similarity in their spatial folds. However, no sequence similarity is detected between GT-A and GT-B families using various alignment approaches (Liu and Mushegian, 2003). This suggests that GT-A and GT-B families are very likely unrelated and evolved independently; and if there is a common ancestor, it must be extremely ancient (Liu and Mushegian, 2003).

1.3.3.3 GT-C Fold

A new fold, GT-C, has recently been suggested and at least nine CAZY families (GT 19, 22, 39, 48, 50, 53, 57, 58 and 59) were proposed to belong to this family (Liu and Mushegian, 2003). All these enzymes except CAZY family 19 (Rosen *et al.*, 2004) consist of large hydrophobic proteins located in the ER or on the plasma membrane, with eight to thirteen predicted multiple transmembrane domains (Liu and Mushegian, 2003). The GT-C family has a conserved N-terminal extra-cytoplasmic loop, where a modified DXD motif (EXD, DXE, DDX, DEX) is present. It remains to be determined if this DXD-like motif in the GT-C family is responsible for binding a divalent metal cation. The catalytic mechanism of the GT-C family is also unknown (Liu and Mushegian, 2003). Enzymes belonging to the GT-C family have been widely found in eukaryotic genomes, absent in archaea, rarely in prokaryotes (so far only UDP-L-arabinose:arabinosyltransferase family) and limited in parasites (Liu and Mushegian, 2003). Most of GT-C fold enzymes are known to utilize dolichyl-phosphate-sugars as donor substrates to synthesize the polysaccharide derivatives of dolichol phosphate (Kikuchi *et al.*, 2003; Liu and Mushegian, 2003).

1.3.3.4 GT-D Fold

The GT-D fold was proposed by Kikuchi *et al.* (Kikuchi *et al.*, 2003) and was categorized into the unclassified fold family by Liu *et al.* (Liu and Mushegian, 2003). According to protein sequence-similarity analysis, α1,2 FucTs (CAZY family 11), α1,6 FucTs (CAZY family 23) and plant xyloglucan α1,2-FucTs (CAZY family 37) do not belong to either GT-A, GT-B or GT-C family (Liu and Mushegian, 2003). As a result they are grouped into the GT-D fold (Kikuchi *et al.*, 2003).

1.3.4 Kinetic Mechanism

A common mechanistic theme is observed in the majority of inverting and retaining enzymes from the resolved crystal structures of twenty-four GTs – a compulsory sequential bi bi system with the sugar donor binding to the enzyme preceeding binding of the acceptor (Qasba *et al.*, 2005; Ramakrishnan *et al.*, 2004). The donor-enzyme binding event gives rise to a conformational change of the enzyme in one or two flexible loops (Table 1-2) thereby its conformation alters from an open to a closed state. Such a conformational change allows the loops to act as a lid to cover the bound donor and to create the binding sites for acceptor. When an acceptor binds to the donor-enzyme complex, its non-reducing end is placed in a position close to the anomeric center of the sugar donor within the catalytic pocket to assure the transfer reaction occurs. The oligosaccharide product is released first, followed by the release of nucleotide portion of the donor. The flexible loops of the enzyme eventually return to their original conformation to allow a new catalytic cycle to begin (Qasba *et al.*, 2005). Enzymes following this mechanism include SpsA (Charnock and Davies, 1999), CstII (Chiu *et al.*, 2004), α1,4 galactosyltransferase LgtC (Persson *et al.*, 2001), Blood group A and B transferases (Patenaude *et al.*, 2002) and β1,4 galactosyltransferase (Gastinel *et al.*, 1999).

It should be noted; however, that some GTs members adopt the compulsory sequential bi bi mechanism where the acceptor binds to the enzyme first followed by the donor molecule such as the two oleandomycin glycosyltransferases, which transfer glucose from UDP-glucose to many macrolides such as oleandomycin, rosaramycin, lankamycin and clarithromycin (Quiros and Salas, 1995; Quiros *et al.*, 2000). Their crystal structures are not yet resolved, and whether or not a conformational change of the enzyme is involved in the catalytic process remains to be determined. Other than the sequential bi bi mechanism, the ping-pong bi bi and the random bi bi mechanisms were predicted for α1,6 FucT (Takahashi *et al.*, 2000) and for α1,2 FucT (Beyer and Hill, 1980; Palcic *et al.*, 1989a), respectively. Again, no crystal structures have been resolved for α1,6 and α1,2 FucT families, so the detailed mechanism remains to be identified.

1.3.5 Determination of Donor Specificity

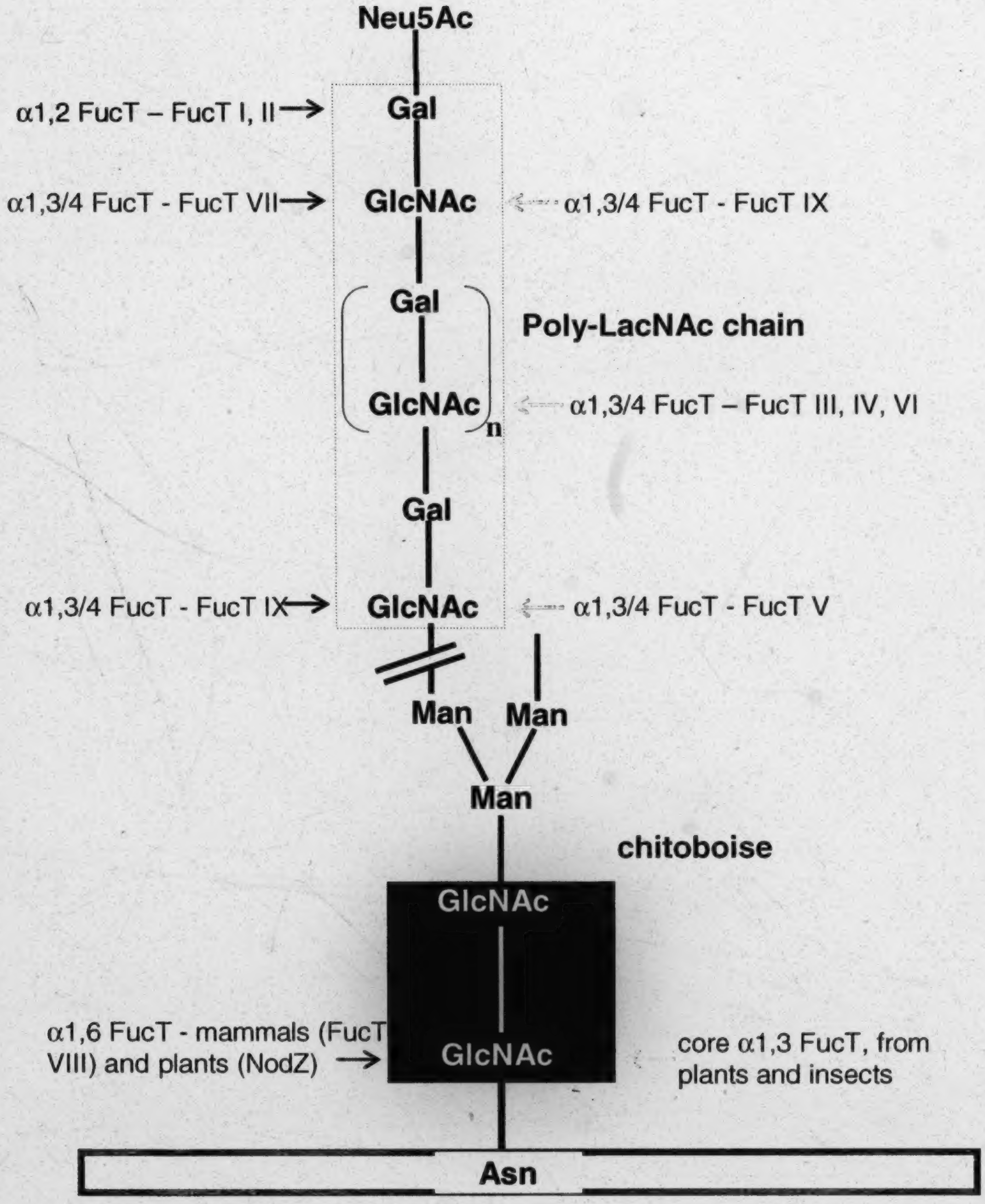
GT enzymes can generally use several different oligosaccharide structures as acceptors efficiently, but are very restrictive in recognition of the donor substrates. The available crystal structures of GTs suggest that just one single amino acid can control the donor specificity. For instance, Tyr^{289} in bovine β1,4 galactosyltransferase (βGalT) determines whether the enzyme recognizes UDP-Gal but not UDP-GalNAc as a donor molecule (Ramakrishnan and Qasba, 2002); His^{308} in human β1,3 glucoronyltransferase (GlcAT-I) restricts the donor specificity to UDP-GlcUA but not UDP-Glc, UDP-

Mannose or UDP-GlcNAc (Kakuda *et al.*, 2004; Ouzzine *et al.*, 2002; Pedersen *et al.*, 2002). The human blood group α1,3 N-acetylgalactosyltransferase A and α1,3 galactosyltransferase B (GTA and GTB) use UDP-GalNAc and UDP-Gal as donor substrate, respectively, and a single amino acid Met^{266} or Leu^{266} in GTA and GTB, respectively, discriminate between these two donor substrates (Marcus *et al.*, 2003).

1.4 Fucosyltransferase Family (α1,2. α1,3/4, α1,6)

FucTs, belonging to the GT superfamily, catalyze the inverting reaction, and are widely expressed in vertebrates, invertebrates, plants and bacteria. The α1,2 FucTs transfer fucose from donor GDP-Fuc to the galactose moiety of Type II (Galβ1,4GlcNAc) or Type I (Galβ1,3GlcNAc) structures at the periphery (non-reducing terminus) site of oligosaccharides. α1,3/4 FucTs transfer fucose from donor GDP-Fuc to the GlcNAc moiety of Type II or Type I structures also at the periphery site of the oligosaccharide acceptors (Fig. 1-5). In contrast to mammalian and bacterial α1,3 FucTs, those from plants including mung bean (Leiter *et al.*, 1999), and *Arabidopsis thaliana* (Strasser *et al.*, 2004; Wilson *et al.*, 2001), and insects (van Tetering *et al.*, 1999) transfer fucose to the Asn-branched GlcNAc moiety of the chitobiose unit on the Asn-linked oligosaccharide acceptor (Fig 1-5). These enzymes should be designated as core α1,3 FucTs. The α1,6 FucTs transfer fucose from donor GDP-Fuc to the innermost GlcNAc moiety on the Asn-linked chitobiose unit of oligosaccharides (Fig. 1-5). Likewise, these enzymes are core α1,6 FucTs, which have been identified in mammals such as pig, rat and human (Miyoshi *et al.*, 1997; Uozumi *et al.*, 1996). The NodZ proteins from Rhizobium species also display α1,6 FucT activity but they prefer chitin oligosaccharides as acceptors, particularly the penta- and hexa-saccharide forms (Quinto *et al.*, 1997).

Fig.1- 5. **Fucosylation of α1,2, α1,3/4 and α1,6 FucTs.** The two main types of acceptor substrates: chitobiose (blue square) and poly N-acetyllactosamine (LacNAc) chain of Type I or Type II structures (framed in the dotted line) and the corresponding fucosyltransferases (FucTs), which are able to add fucose in α1,2, α1,3 and α1,6 linkages (Oriol *et al.*, 1999). The preferred fucosylation sites within the poly-LacNAc chain for α1,3 FucTs IV, V, VI, VII and IX is given. Of note, FucT VII is only able to use the sialylated-Type II acceptor (in purple). With the sialylated-Type II acceptor, FucT IX switches the preferred fucosylation site from the terminal end to the innermost position (in purple).

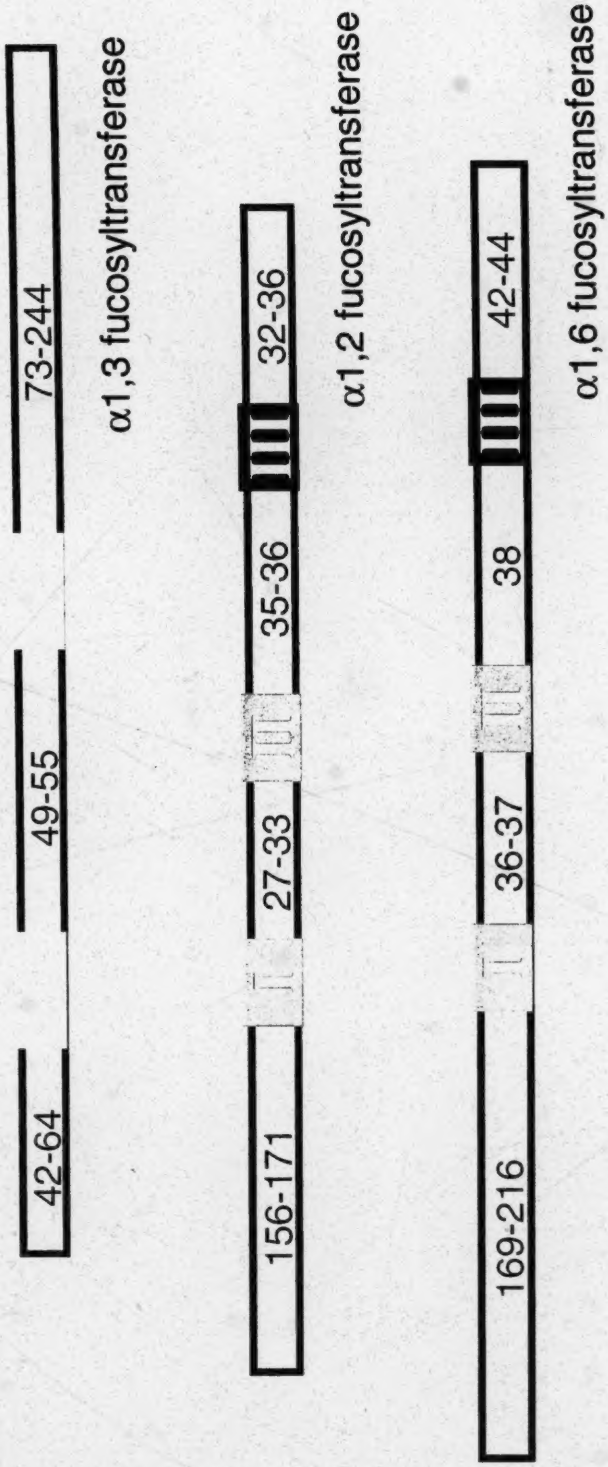

Neu5Ac
α1,2 FucT – FucT I, II
Gal
α1,3/4 FucT - FucT VII
GlcNAc
α1,3/4 FucT - FucT IX
Gal
Poly-LacNAc chain
GlcNAc
n
α1,3/4 FucT – FucT III, IV, VI
Gal
α1,3/4 FucT - FucT IX
GlcNAc
α1,3/4 FucT - FucT V
Man
Man
Man
chitoboise
GlcNAc
α1,6 FucT - mammals (FucT VIII) and plants (NodZ)
GlcNAc
core α1,3 FucT, from plants and insects
Asn

NodZ proteins share less than 20% homology with other α1,6FucTs from vertebrates and invertebrates (Oriol *et al.*, 1999).

Despite the sequence homology within each subfamily, α1,2 FucTs, α1,3/4FucTs and α1,6 FucTs share extremely low primary sequence identity. Woodcock *et al.* reported an alignment method called hydrophobic cluster analysis (HCA), which is able to detect and compare the hydrophobic clusters in each protein. Such hydrophobic clusters are presumed to coincide with the regular secondary structure elements that constitute the hydrophobic core of globular proteins. As a result, this method is efficient in capturing the structural similarity beyond the very low primary sequence identity, provided the proteins adopt similar folding conformation (Woodcock *et al.*, 1992).

Using HCA analysis to align FucTs from vertebrates, invertebrates, plants and bacteria, six FucT motifs have been identified (Oriol *et al.*, 1999) (Fig. 1-6). Two in the catalytic region shared by α1,2 and α1,6 FucT families, implying that these two families may share a common ancestor. The α1,6 FucTs seem to have evolved much more slowly than the α1,2 FucTs. Only 2-5% rather than 20-30% sequence nonidentity is observed among different vertebrate α1,6 FucTs versus the α1,2 FucTs, respectively (Javaud *et al.*, 2000). Two FucT motifs are unique for α1,3 FucTs, one is specific for α1,2 FucT and another is specific for α1,6 FucT (Oriol *et al.*, 1999) (Fig 1-6). Although α1,3 FucTs lack the consensus peptide segment that is present in α1,2 and α1,6 FucTs, it appears that the first common motif shared by α1,2 and α1,6 FucTs displays similar hydrophobic features and conserved basic and acidic residues with the first α1,3 FucT motif (Breton *et al.*, 1996, 1998).

Fig. 1-6. Schematic representations of α1,3, α1,2 and α1,6 fucosyltransferses showing the location of the conserved motifs. The light and dark blue rectangles I, II and III identify the motifs that are unique to each subfamily of fucosyltransferases. The green rectangles I and II identify the motifs shared by both α1,2 and α1,6 fucosyltransferases. Numbers inside the rectangles indicate the range of pre-, post- and inter-motifs peptide lengths in bacterial fucosyltransferases. Adapted from (Oriol *et al.*, 1999).


α1,3 fucosyltransferase
73-244
49-55
42-64
α1,2 fucosyltransferase
32-36
35-36
27-33
156-171
α1,6 fucosyltransferase
42-44
38
36-37
169-216

To date, there is not enough evidence to suggest that α1,3/4 FucTs share a common ancestor with α1,2 and α1,6 FucT subfamilies. Oriel *et al.* suggested that if there were a common ancestor, the current plant and insect core α1,3 FucTs and α1,6 FucTs, which use chitobiose as acceptor substrates, might be the present forms of the ancient ancestral gene. Chitobiose is present in the first polymannose oligosaccharide block, whereas additions of the GlcNAc and galactose moieties constituting the terminal LacNAc (where the fucose transfer takes place by α1,2 and α1,3 FucTs) are the last events of oligosaccharide chain elongation (Fig. 1-5). This suggests the α1,2 and α1,3 FucT families should be more recent in evolution than the enzymes using chitobiose as substrate (Oriol *et al.*, 1999).

1.4.1 Mammalian FucTs

Nine human FucTs have been characterized to date that have either α1,2, α1,3/4 or α1,6 activity. Human α1,2 and α1,3/4 FucTs are involved in the last steps of the synthesis of A, B, H and Lewis-related carbohydrate antigens. The genes *fucT1*, *fucT2* and *sec1* (a pseudogene with a mutation causing frameshift) encode the H, Se (secretor), and the non-functional α1,2 FucTs, respectively. *fucT3* to *fucT7* genes encode α1,3 FucTs, FucT III to FucT VII, of which FucT III and FucT V also have α1,4 activity. *fucT8* encodes an α1,6 FucT, FucT VIII, which is involved in the synthesis of N-glycans (Miyoshi *et al.*, 1999; Yamaguchi *et al.*, 1999; Yamaguchi *et al.*, 2000). Each *fut* gene is located at a different chromosomal locus (Table 1-3) except that *fucT3*, *fucT5* and *fucT6* are organized in a cluster within 1 centimorgan in the short arm of chromosome 19 band 19p13.3 (Costache *et al.*, 1997, Oriol, 1999 #1). Nine human *fut* genes have distinctive tissue distribution, which is summarized in Table 1-3. It is worth mentioning that FucT

Table 1-3. The gene loci and tissue distribution of the genes *fut1* to *fut9* and the enzyme activities, acceptor substrate specificities and preferred fucosylation sites of human α1,2 and α1,3/4 fucosylstransferases, FucT 1-FucT IX.

Gene	Gene loci & # of exons	Enzyme	Activity	Tissue Distribution	Preferred Fucosylation Sites of Using the Neutral Poly-LacNAc Chain	Substrate Specificity
fut1	19q13.3 One exon	FucT 1	α1,2	Erythrocyte membrane vascular endothelium (Mollicone *et al.*, 1995).	N/A	N/A
fut2	19q13.3 One exon	FucT 2	α1,2	Epithelial cells and in body fluids such as saliva (Avent, 1997).	N/A	N/A
fut3	19p13.3 One exon	FucT III (Lewis type)	α1,3 α1,4	Mainly in exocrine secretions, also abundant in gastrointestinal tissues, but less in spleen, lung, kidney and cervix uteri (Cameron *et al.*, 1995; Kaneko *et al.*, 1999).	Can incorporate multiple fucose residues into poly-LacNAc structures (Holmes and Levery, 1989) and prefers internal over distal site (Holmes and Levery, 1989).	Utilizes both neutral Type I and Type II acceptors, regardless of the terminal galactose linked with α1,2-fucose, α2,3- or α2,6-sialic acid (Holmes and Levery, 1989).
fut4	11q21 One exon	FucT IV (myeloid type)	α1,3	Ubiquitously expressed in all tissues, particularly in leukocytes and brain (Cameron *et al.*, 1995; Homeister *et al.*, 2001; Huang *et al.*, 2000; Kaneko *et al.*, 1999; Weninger *et al.*, 2000).	Favors the intermediate site over the distal and proximal sites (Niemela *et al.*, 1998).	Transfers fucose more efficiently to neutral rather than sialylated Type II acceptors (Sherwood *et al.*, 2002).
fut5	19p13.3 One exon	FucT V	α1,3 α1,4	Minimally expressed in spleen and in restricted quantities in liver, colon, and testes (Cameron *et al.*, 1995; Kaneko *et al.*, 1999).	Shows high, intermediate and low transfer at the proximal, mid-chain, and distal sites of LacNAc, respectively (Pykari *et al.*, 2000).	Prefers both fucosylated and sialylated Type II acceptors but does not favor sialylated Type I structures (de Vries *et al.*, 1995).
fut6	19p13.3 One exon	FucT VI (plasma type)	α1,3	Present in plasma, renal proximal tubules and hepatocytes (Cameron *et al.*, 1995; Kaneko *et al.*, 1999).	Unable to initiate fucose transfer to the internal site of an extended LacNAc chain (Weston *et al.*, 1992); prefers the internal to the distal site (Nishihara *et al.*, 1999).	Utilizes both neutral Type II and sialylated Type II acceptors with high efficiency (de Vries *et al.*, 1995; De Vries *et al.*, 1997; Weston *et al.*, 1992).
fut7	9q34.3 One exon	FucT VII (leukocyte type)	α1,3	Abundantly in leukocytes and the high endothelial cells of the venule (Homeister *et al.*, 2001; Huang *et al.*, 2000; Mollicone *et al.*, 1995; Weninger *et al.*, 2000).	Only transfers fucose to sialylated but not to neutral LacNAc chains. With sialylated LacNAc-acceptors with multiple potential fucosylation sites, it generally transfers only one fucose residue to the GlcNAc residue nearest to the negatively charged sialic acid (Stroud and Holmes, 1997).	Transfers fucose to the sialylated Type II acceptors but not to the neutral Type II or 3'-sulfated Type II acceptors (Britten *et al.*, 1998; de Vries *et al.*, 2001b).
fut8	14q23 Nine exons	FucT VIII	α1,6	Ubiquitously expressed (Martinez-Duncker *et al.*, 2004).	N/A	N/A
fut9	6q16 One exon	FucT IX	α1,3	Primarily in brain, less expressed in stomach, spleen, kidney and peripheral blood cells (de Vries *et al.*, 2001a; Nishihara *et al.*, 2003).	Strongly prefers the distal sites on neutral Type II acceptor. It shifts the preference to the innermost LacNAc unit when sialylated poly-LacNAc chain is used as an acceptor (Toivonen *et al.*, 2002).	Transfers efficiently to neutral Type II acceptor, very poorly to sialylated Type II disaccharide but efficiently to the sialylated poly-LacNAc chains (Toivonen *et al.*, 2002).

IV and FucT VII are expressed in leukocytes where they are responsible for generation of the functional selectin ligands (Homeister *et al.*, 2001; Lowe, 2003).

The majority of the coding sequences (cds) of vertebrates *fucT* genes are monoexonic, with the exception of *fucT 7* that has two exons in human and mouse, and *fucT8* with at least nine or five exons in human and bovine, respectively (Table 1-3) (Javaud *et al.*, 2000; Oriol *et al.*, 1999). All eighteen of *Caenorhabditis elegans* putative FucT genes contain multiple (5 to 15) exons (Oriol *et al.*, 1999). As a major evolutionary trend is the loss of introns and the fusion of exons thereby forming more complicated exons, it is expected that the most ancient genes harbor split cds and more recent genes have monoexonic cds (Javaud *et al.*, 2003). This suggests the α1,6 FucT genes are the progenitors with their appearance preceeding the α1,2 and α1,3 FucT gene families. Mammalian molecular phylogeny tree analysis and the gene structural comparison suggest, bovine *fucT* is likely the ancestral orthologous homologue of the *fucT3-fucT5-fucT6* gene cluster (Oulmouden *et al.*, 1997; Wierinckx *et al.*, 1999).

1.4.2 Mammalian α1,3/4 FucTs

Currently, despite no available crystal structures haven been reported for any members of the FucT family, extensive investigations have been carried out on structure-function relationship of mammalian α1,3/4 FucTs and this has greatly advanced the understanding of the mechanistic basis of this enzyme family.

1.4.2.1 Domain Construction

In the same way as many other Golgi resident GTs, mammalian α1,3/4 FucT are Type II transmembrane proteins containing an N-terminal cytoplasmic tail (6-12 amino acids), a transmembrane domain (17-20 amino acids) and an extended stem region followed by a large globular C-terminal catalytic domain (Fig 1-4). Creation of truncated constructs helped to delineate the minimal catalytic domain of human FucTs (Xu *et al.*, 1996). Sixty-one and seventy-five amino acids could be deleted from the N-terminus in human FucT III and V, respectively, without a significant loss of enzyme activity. In contrast, removal of one or more amino acids from the C-terminus causes a dramatic or total loss of enzyme activity (Xu *et al.*, 1996).

1.4.2.2 Domain Controling Type I Acceptor Specificity

FucT III, V and VI share 85% sequence identity. Bovine FucT, being orthologous to human FucT III, V and VI, shares about 70% protein sequence identity (Oulmouden *et al.*, 1997; Weston *et al.*, 1992). Notably, these four FucTs display distinctive acceptor specificities. FucT VI and bovine FucT can only use Type II acceptors so display α1,3 activity, whereas human FucT III and V can use both Type I and Type II acceptors exhibiting both α1,3 and α1,4 activities but with unique preferences. FucT III favors Type I acceptors therefore it is predominantly an α1,4 FucT, whereas FucT V prefers Type II acceptors making it primarily an α1,3 FucT (de Vries *et al.*, 2001a and references therein).

To identify which domain of human FucT III, V, VI and bovine FucT confers the broader or narrower acceptor substrate usage, twenty chimeras derived from human FucT III and VI, and one chimera from human FucT V and VI, were constructed by domain swapping. The ability of each construct to utilize Type I and Type II substrates for fucose transfer was examined. Data show that eleven distinctive amino acids at the N-terminal variable region, specifically within FucT $\text{III}^{105\text{-}151}$ (corresponding to FucT $\text{V}^{117\text{-}163}$ and FucT $\text{VI}^{104\text{-}150}$) control Type I acceptor specificity (Legault *et al.*, 1995). When a single amino acid, Trp^{111}, of FucT III was replaced with an Arg, FucT III prefers Type II over Type I acceptors. Arg^{115} is present in bovine FucT at the equivalent position to Trp^{111} in human FucT III. The adjacent residue Asp^{112} in FucT III, corresponding to Glu^{116} in bovine FucT, is able to further modulate the relative Type I and Type II acceptor specificity (Dupuy *et al.*, 1999) (Fig. 1-7). Detailed site-directed mutagenesis of Trp^{111} in FucT III showed that substitution with any non-aromatic residues greatly reduced α1,4 activity (<20%), whereas replacement with an aromatic residue (Tyr or Phe), retains the α1,4 activity to least 50% of wild type level (Dupuy *et al.*, 2004). This suggests that the aromatic nature of the Trp residue is essential for Type I substrate specificity. This Trp residue is highly conserved in other mammalian FucTs containing a substantial level of α1,4 activity, such as α1,4 FucTs from chimpanzee (Costache *et al.*, 1997), rhesus macaque (Old World Monkey) (Dupuy *et al.*, 2002) and plants (Bakker *et al.*, 2001; Leonard *et al.*, 2002). Remarkably, at the corresponding position, Arg is conserved in human FucTs with exclusive α1,3 activity including FucT IV, VI and VII (Dupuy *et al.*, 1999).

In a separate investigation, human FucT III and V were used as parental FucTs to construct chimeras by domain swapping. The data showed that eight amino acids within the N-terminal hypervariable stem region of FucT $III^{62\text{-}110}$ (corresponding to FucT $V^{76\text{-}123}$) controls the Type I acceptor preference (Xu *et al.*, 1996). Site-directed mutagenesis data showed that, replacement of $Asn^{86}Thr^{87}$ in FucT V with $His^{87}Ile^{88}$ of FucT III increased α1,4 activity (Nguyen *et al.*, 1998) (Fig. 1-7).

On the other hand, a single amino acid substitution at the C-terminus of human FucT III and V (Asp^{336} in FucT III and Ala^{349} in FucT V) also altered the acceptor and donor

```
                        10        20        30        40        50        60
                         |         |         |         |         |         |
hFucT_IV        MGAPWGSPTAAAGGRRGWRRGRGLPWTVCVLAAAGLTCTALITYACWG----------QL
hFucT_VII       MNNAGHGPT----RR---LRGLGVLAGVALLAA--L-------WLLW-----------LL
hFucT_III       MDPLGAAKPQWPWRRCLAALLFQLLVAVCFFSYLRVSRDDATGSPR-----------APS
hFucT_V         MDPLGPAKPQWLWRRCLAGLLFQLLVAVCFFSYLRVSRDDATGSPRPGLMAVEPVTGAPN
hFucT_VI        MDPLGPAKPQWSWRCCLTTLLFQLLMAVCFFSYLRVSQDDPTVYPN--------------
bFucTx3         MYPPGCAKVKCSWHHCLPGLLLQLLLALCFFSYLRMSQEKPKPKPMWVS---------EL
hFucT_IX        MTSTSKGILRPFLIVCIILGCFMACLLIYIKPTNSWIFSPMESASS------------VL
                *   .                       :  .  .

                        70        80        90       100       110       120
                         |         |         |         |         |         |
hFucT_IV        PPLPWAS--PTPSRPVGVLLWWEPFGGRDSAPRPPPDCPLRFNISGCRLLTDRASYGEAQ
hFucT_VII       GSAPRGT--PAPQPTITILVWHWPFT--DQPPELPSDTCTRYGIARCHLSANRSLLASAD
hFucT_III       GSSRQDT--TPTRPTLLILLWTWPFH---IPVALSRCSEMVPGTADCHITADRKVYPQAD
hFucT_V         GSRCQDSMATPAHPTLLILLWTWPFN---TPVALPRCSEMVPGAADCNITADSSVYPQAD
hFucT_VI        GSRFPDSTGTPAHSIPLILLWTWPFN---KPIALPRCSEMVPGTADCNITADRKVYPQAD
bFucTx3         GAPSQATEGSSAHLPLRVLLWTWPFN---QPVALSRCSELWPGTADCQLTVNRSEYPQAD
hFucT_IX        KMKNFFSTKTDYFNETTILVWVWPFG---QTFDLTSCQAMFN-IQGCHLTTDRSLYNKSH
                                :*:*  **       .   .             *.:  .:     .:.
                     .

                       130       140       150       160       170       180
                         |         |         |         |         |         |
hFucT_IV        AVLF[illegible]RDLVKGPPDWPPPWGIQAHTAEEVDLRVLDYEEAAAAAEALATSSPRPPGQRWV
hFucT_VII       AVVF[illegible]RELQTRRSHLPLA-------------------------QRPRGQPWV
hFucT_III       TVIV[illegible]WDIMSNPKSRLPP-------------------------SPRPQGQRWI
hFucT_V         AVIV[illegible]WDIMYNPSANLPP-------------------------PTRPQGQRWI
hFucT_VI        AVIV[illegible]REVMYNPSAQLPR-------------------------SPRRQGQRWI
bFucTx3         AVLV[illegible]REVSHRPQMQLPP-------------------------SPRPPGQRWV
hFucT_IX        AVLI[illegible]RDISWDLTNLPQQ--------------------------ARPPFQKWI
                :*:.** ::                                          *   * *:

                       190       200       210       220       230       240
                         |         |         |         |         |         |
hFucT_IV        WMNFESPSHSPGLRSLASNLFNWTLSYRA[illegible]VFVPYGYLYPRSHPGDPPSGLAPPLSR[illegible]
hFucT_VII       WASMESPSHTHGLSHLRG-IFNWVLSYRR[illegible]IFVPYGRLEPHWGP-------SPPLPA[illegible]
hFucT_III       WFNLEPPPNCQHLEALDR-YFNLTMSYRS[illegible]IFTPYGWLEPWSGQ---PAHPPLNLSA[illegible]
hFucT_V         WFSMESPSNCRHLEALDG-YFNLTMSYRS[illegible]IFTPYGWLEPWSGQ---PAHPPLNLSA[illegible]
hFucT_VI        WFSMESPSHCWQLKAMDG-YFNLTMSYRS[illegible]IFTPYGWLEPWSGQ---PAHPPLNLSA[illegible]
bFucTx3         WFSMESPSNCLKLKDLDG-YFNLTMSYRR[illegible]IFMPYGWLEPWPSQ---PVETLLNISA[illegible]
hFucT_IX        WMNLESPTHTPQKSGIEH-LFNLTLTYRR[illegible]IQVPYGFLTVSTNP------FVFEVPS[illegible]
                * .:*.*.:        :    **  .::** ***:  *** *                   :. *

                       250       260       270       280       290       300
                         |         |         |         |         |         |
hFucT_IV        QGLVAWVVSHWDERQARVRYYHQLSQHVTVDVFGRGGPGQPVPEIGLLHTVARY[illegible]FYLAF
hFucT_VII       SRVAAWVVSNFQERQLRARLYRQLAPHLRVDVFGRAN-GRPLCASCLVPTVAQY[illegible]FYLSF
hFucT_III       TELVAWAVSNWKPDSARVRYYQSLQAHLKVDVYGRSH--KPLPKGTMMETLSRY[illegible]FYLAF
hFucT_V         TELVAWAVSNWKPDSARVRYYQSLQAHLKVDVYGRSH--KPLPKGTMMETLSRY[illegible]FYLAF
hFucT_VI        TELVAWAVSNWGPNSARVRYYQSLQAHLKVDVYGRSH--KPLPQGTMMETLSRY[illegible]FYLAF
bFucTx3         TKLVAWVVSNWNTDSIRVQYYKLLKPHLQVDVYGRFH--TPLPHALMAKQLSQY[illegible]FYLAF
hFucT_IX        EKLVCWVVSNWNPEHARVKYYNELSKSIEIHTYGQAF-GEYVNDKNLIPTISAC[illegible]FYLSF
                  :..*.**::      *.: *. *     : :..:*:     :     :    ::  :***:*

                       310       320       330       340       350       360
                         |         |         |         |         |         |
hFucT_IV        ENSQHLDYITE[illegible]LWRNALLAGAVPVVLGPDRANYERFVPRGAFIHVDDFPSASSLASYLL
hFucT_VII       ENSQHRDYITE[illegible]FWRNALVAGTVPVVLGPPRATYEAFVPADAFVHVDDFGSARELAAFLT
hFucT_III       ENSLHPDYITE[illegible]LWRNALEAWAVPVVLGPSRSNYERFLPPDAFIHVDDFQSPKDLARYLQ
hFucT_V         ENSLHPDYITE[illegible]LWRNALEAWAVPVVLGPSRSNYERFLPPDAFIHVDDFQSPKDLARYLQ
hFucT_VI        ENSLHPDYITE[illegible]LWRNALEAWAVPVVLGPSRSNYERFLPPDAFIHVDDFQSPKDLARYLQ
bFucTx3         ENSLHPDYITE[illegible]LWKNALQAWAVPVVLGPSRVNYEQFLPPKAFIHVEDFQSPKDLAQYLL
hFucT_IX        ENSIHKDYITE[illegible]LY-NAFLAGSVPVVLGPSRENYENYIPADSFIHVEDYNSPSELAKYLK
                *** * ******:: **: * :******* * .** ::*  :*:**:*: *. .** :*
```

```
                        370       380       390       400       410
                         |         |         |         |         |
hFucT_IV        FLDRNPAVYRRYFHWRRSYAVHITSFWDEPWCRVCQAVQRAGDRPKSIRNLASWFER
hFucT_VII       GMNE--SRYQRFFAWRDRLRVRLFTDWRERFCAICDRYPHLP-RSQVYEDLEGWFQA
hFucT_III       ELDKDHARYLSYFRWRETLRPRSFS-WAL[illegible]FCKACWKLQQES-RYQTVRSIAAWFT-
hFucT_V         ELDKDHARYLSYFRWRETLRPRSFS-WAL[illegible]FCKACWKLQQES-RYQTVRSIAAWFT-
hFucT_VI        ELDKDHARYLSYFRWRETLRPRSFS-WALAFCKACWKLQEES-RYQT-RGIAAWFT-
bFucTx3         ALDKDYASYLNYFRWRETLRPRSFS-WALMFCKACWKLQQEP-RYQTVPSIASWFQ-
hFucT_IX        EVDKNNKLYLSYFNWRKDFTVNLPRFWESHACLACDHVKRHQ-EYKSVGNLEKWFWN
                 ::.    *  :* **     .    *    *  *    .   . :   .:  **
```

Fig. 1-7. Alignment of human α1,3/4 fucosyltransferases (FucTs), FucT III (gi121137), IV (gi1730133), V (gi1730135), VI (gi1730136), VII (gi1730137), IX (gi55584012) and bovine FucT (gi2851674). Sequence alignment was performed using ClustalW PBIL (Network Protein Sequence Analysis). Residues identical among all FucT examined are in red and marked with an asterisk; highly conserved residues are in green and are marked with a colon; partially conserved residues are in blue and marked with a period, and divergent residues are in black. Two highly conserved α1,3 FucT motifs are shaded in light gray. The residues at the stem region that confers Type I acceptor specificity are shaded in yellow. The residues (Cys, Ser or Thr) responsible for donor binding are shaded in light blue. The three highly conserved Lys residues are shaded in blue. Cys residues in human FucT III and FucT VII that form disulfide bonds are shaded in green. The C-terminal Asp/Ala residue of human FucT III and V that is involved in donor and acceptor binding is shaded in red. A highly conserved DXD motif is located within the α1,3FucT motif I and is highlighted in black.

kinetics (Fig. 1-7). When Asp^{336} in FucT III was replaced with Ala, the enzyme displayed a 40-fold reduction in affinity for H-Type I acceptor and a 4-fold decrease in affinity for donor GDP-Fuc. In contrast, when Ala^{349} of FucT V was substituted with Asp, FucT V becomes more active with increased affinity for H-Type II acceptor and an overall 8-fold increase in the catalytic efficiency (Vo *et al.*, 1998). Thus, the residues at both N- and C-terminus of human FucTs are involved in conferring substrate specificity and affinity, suggesting that the FucT enzyme may adopt a fold bringing the N- and C-terminal segments close together to form a catalytic binding pocket.

1.4.2.3 Acceptor Specificities

Human FucT III, IV, V, VI, VII and IX transfer fucose not only to Type II (and Type I for FucT III and V) disaccharides, but also to acceptors containing a Type II poly-LacNAc chain. Nevertheless, these six human α1,3/4 FucTs display different preferred fucosylation sites (Fig. 1-5 and Table 1-3). In addition, they can also use 3'-sialylated or 2'-fucosylated Type II or Type I structures as acceptor, again with distinctive efficiencies (Table 1-3).

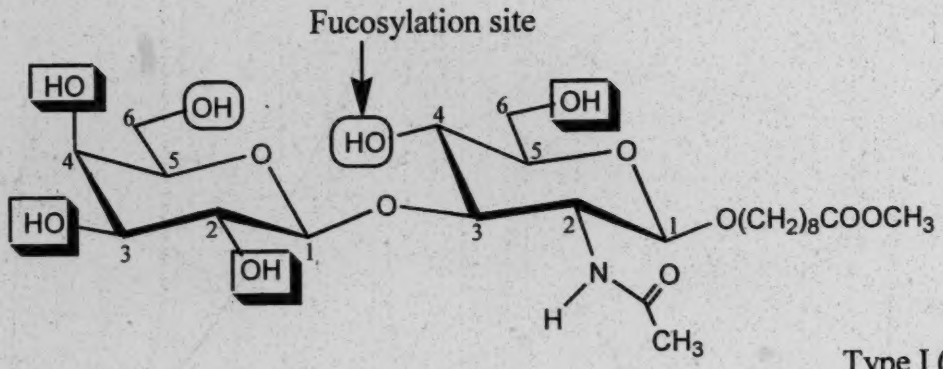
1.4.2.4 Acceptor Key Polar Group Mapping

Lemieux's pioneering studies have shown that oligosaccharide-protein recognition occurs through hydrogen bond interactions between residues on the enzyme and the hydroxyl groups on the carbohydrate acceptors. Such hydroxyl groups play essential roles in recognition by proteins and are designated as key polar groups (Lemieux, 1989, 1993, Spohr *et al.*, 1991). The key polar groups of Type II and Type I disaccharide acceptors have been mapped for human FucT III, IV, V (de Vries *et al.*, 1995) and VI (De Vries *et al.*, 1997), by employing a series of modified and deoxygenated Type II and Type I analogues. For human FucT III, IV V and VI (Breton *et al.*, 1998; de Vries *et al.*, 1995; De Vries *et al.*, 1997), every hydroxyl group, except the 6-OH of galactose and the reactive hydroxyl at C-3 or C-4 of GlcNAc in Type II or Type I acceptors, respectively, can tolerate the modifications. Deoxygenation at C-6 of galactose completely abolished enzyme activity, and dexoygenation at C-3 or C-4 of GlcNAc in Type II or Type I acceptors rendered the compound neither an acceptor nor an inhibitor (Breton *et al.*, 1998; de Vries *et al.*, 1995; De Vries *et al.*, 1997). A similar study has been performed for human milk α1,3 and α1,3/4 FucTs, where the same results were reported, except that the deoxygenation at C-4 of the GlcNAc in Type I acceptor creates a very weak inhibitor with K_i at 9 mM (Gosselin and Palcic, 1996) or K_i at 38 mM (Du and Hindsgaul, 1996). From these studies it was concluded that, the OH-6 of galactose and the reactive hydroxyl groups in Type II and Type I act as key polar groups that are required for recognition by human α1,3/4 FucTs (Fig. 1-8). During the key polar group mapping studies, it was reported that the 2-acetamido group of LacNAc is required for the optimal activity with human FucT III, IV and V (de Vries *et al.*, 1995).

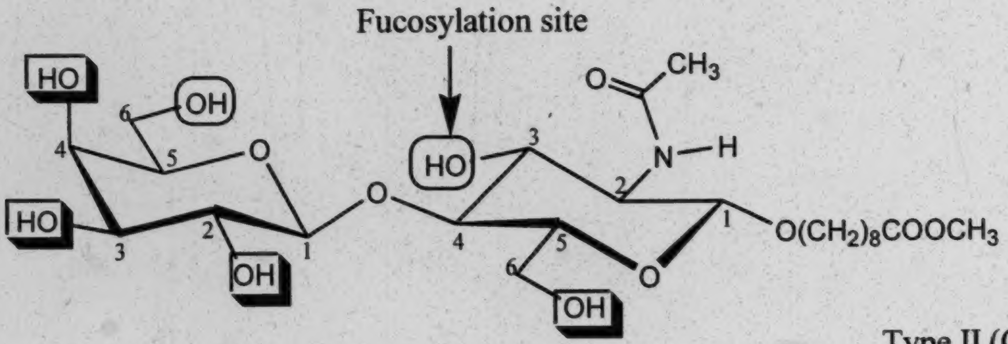
1.4.2.5 Donor Binding Sites

Results from protein sequence alignments show that a conserved Cys residue is present in FucT III, V and VI (Cys^{143} in FucT III, Cys^{156} in FucT V and Cys^{142} in FucT VI), but is absent in human FucT IV and VII, in which a serine or threonine (Ser^{178} in FucT IV and Thr^{127} in FucT VII) is found at the corresponding position (Fig. 1-7). FucT III, V and VI are sensitive to N-ethylmaleimide (NEM, a sulfhydryl-group modifying reagent) treatment but FucT IV and VII are resistant to the treatment (Holmes *et al.*, 1995). FucT III, V and VI can be effectively protected from NEM inactivation by

Fig. 1-8. Type I and Type II acceptors with key polar groups that are essential for recognition by human α1,3/4 fucosyltransferases III, IV, V and VI and human α1,3/4 fucosyltransferases from human milk.



Type I (Galβ1,3GlcNAc)



Type II (Galβ1,4GlcNAc)

▭ Modifications tolerated

◯ Key polar groups

pretreatment of the enzyme with GDP-Fuc and GDP, but not UDP-galactose, fucose or LacNAc, suggesting that the sulfhydryl group on FucT III and V is either directly involved in binding the nucleotide portion of the donor substrate or is in close proximity to the binding pocket. When Ser^{178} of FucT IV is substituted with Cys, the enzyme becomes sensitive to the NEM treatment and inactivation is also prevented by the GDP-Fuc pretreatment. This suggests that the highly conserved Cys residue in FucT III, V and VI and the Ser and Thr residues at the corresponding position in FucT IV and VII respectively, is either very close to or part of the donor binding pocket (Holmes *et al.*, 1995). It was also shown that α1,3/4 FucT isolated from human small cell lung carcinoma NCl-H69 cells was sensitive to treatment by pyridoxal 5'-phosphate/NaBH4 (specifically modifies Lys) and GDP-Fuc pretreatment also protected inactivation, suggesting Lys residues, with their positions yet to be defined, might also be involved in donor binding (Holmes, 1992).

1.4.2.6 Disulfide Bonds Patterns

There are several conserved Cys residues in human α1,3/4 FucTs, therefore, the disulfide bond patterns have been investigated. Human FucT III and VII exhibit distinctive patterns of disulfide bonds: two disulfide bonds form between Cys^{81} to Cys^{338} and Cys^{91} with Cys^{341} in FucT III (Holmes *et al.*, 2000) versus three disulfide bonds between Cys^{68} to Cys^{76}, Cys^{211} with Cys^{214} and Cys^{318} with Cys^{321} to generate three small loops in FucT VII (de Vries *et al.*, 2001c) (Fig. 1-7). The disulfide bond pattern can be used as a criterion for identifying if a predicted 3D structural model is appropriate (de Vries *et al.*, 2001a).

1.4.2.7 Other Critical Residues

In addition to identifying residues that discriminate Type II and Type I acceptor structures and that recognize GDP-Fuc donor molecule, a number of other critical residues have been shown to play a role in enzyme catalysis. To determine which residues are essential for enzyme activity, chemical modification is a commonly used methodology to specifically modify certain amino acids and monitor the alterations of enzyme activities. Such chemical modifucatuib reagents include NEM (specifically modifies Cys), DEPC (specifically modifies His), n-bromosuccinimide (specifically modifies Trp), pyridoxal 5'-phosphate/NaBH4 (specifically modifies Lys) and phenylglyoxal (specifically modifies Arg). FucT III is found to be sensitive to the treatment with DEPC and n-bromosuccinimide, but resistant to phenylglyoxal, indicating that His and Trp residues, but not Arg, are essential for human FucT III activity (Holmes *et al.*, 1995; Leonard *et al.*, 2005). Likewise, chemical modification of α1,4 FucT from the plant *Silene alba* shows that His and Trp but not Arg, Lys and Cys residues are critical for its α1,4 activity (Leonard *et al.*, 2005). The disadvantage of chemical modification is that this method may fail to identify the exact location of the critical residue if more than one amino acid of a particular type is present in the enzyme.

Human α1,3/4 FucT alignments reveal three lysine residues conserved in FucT III, IV, V and VI. In FucT VII, only two are present and can be aligned with the first and the third lysine residues in FucT III, IV, V and VI. Arg^{223} occupies the position corresponding to the second Lys (Sherwood *et al.*, 1998) (Fig. 1-7). Mutation of this second conserved Lys residue (Lys^{255}) in FucT V to Arg dramatically decreases the enzyme activity, and replacement of Lys^{255} with Ala completely abolishes the activity. This suggests that a basic residue at position 255 is required for FucT V enzyme activity.

In contrast, mutation of Arg^{223}→Lys of FucT VII increases the enzyme activity without modifying the donor and the acceptor binding affinity. Using the Lys and Arg modifying reagents, pyridoxal 5'-phosphate/NaBH4 and phenylglyoxal respectively, human FucT V and FucT VII become inactive. However pretreatment with GDP-Fuc fails to protect against inactivation, indicating that neither Lys^{255} in FucT V nor Arg^{223} in FucT VII is localized close to the donor binding sites (Sherwood *et al.*, 1998).

A pair of His residues adjacent to the region that determines Type I substrate specificity (i.e. $Trp^{111}Asp^{112}$ of FucT III and $Arg^{115}Glu^{116}$ of bovine FucT) (Fig. 1-7) is highly conserved in mammalian, chicken and fish FucTs. Site-directed mutagenesis studies of $His^{113}His^{114}$ in FucT IV showed that modification of His^{113} either completely inactivated the enzyme or dramatically reduced the activity, whereas mutation of His^{114} only slightly reduced the activity. Neither mutation caused any changes in donor binding. Mutation of His^{114} increased the K_m s for both Type II and H-Type II acceptors with the latter less dramatically. This implies that this His-His motif might be involved in acceptor binding and perhaps interacts with the GlcNAc moiety (Sherwood *et al.*, 2002). Of note, this His-His motif is not conserved in FucTs from plants, bacteria and invertebrates (i.e. *C. elegans* and *Schistosoma mansoni*) (Sherwood *et al.*, 2002 and references therein).

Lastly, every amino acid within the second α1,3 FucT motif (^{240}YKFLFENSYTEK^{258}) (Fig. 1-7) of human FucT VI has been mutated to alanine and the effect on enzyme activity and donor and acceptor binding has been determined. Every mutation reduces the enzyme activity to variable degrees. Most of the mutations affect donor binding except Ser^{249}→Ala, which increases the Km for acceptor. This confirms that each amino acid within this motif region is likely engaged in either substrate binding or enzyme catalysis (Jost *et al.*, 2005).

1.4.2.8 Metal Binding

Currently it is not known if the α1,3/4 FucT family requires a divalent metal cation for enzyme catalysis or simply the metal cation facilitates the reaction. Experiments show that FucT V prefers Mn^{2+}, but can also use Ca^{2+}, Co^{2+} and Mg^{2+} (Murray *et al.*, 1996). The α1,4 FucT from *Silene alba* seems to require Mg^{2+} or Mn^{2+} for full activity (Leonard *et al.*, 2005). Some evidence suggests that α1,2 and α1,6 FucTs do not require the metal cofactor for catalysis (Miyoshi *et al.*, 1999; Takahashi *et al.*, 2000).

1.4.2.9 Catalytic Mechanisms

At present, there are only three studies describing the mechanism of fucose transfer by FucTs and all examined human FucT V (Murray *et al.*, 1996; Murray *et al.*, 1997; Qiao *et al.*, 1996). The FucT V-catalyzed mechanism was examined using the inhibitor azatrisaccharide, which was synthesized by linking the α-fucosidase inhibitor, β-L-homofuconojirimycin, to the 3-OH of LacNAc via an ethylene spacer. The inhibitory effect of this aza sugar on FucT V as a function of acceptor LacNAc was modest. However, the presence of GDP (30 μM) dramatically reduced the K_i value of the aza sugar by 77-fold. This indicated that the GDP and the aza sugar likely form a complex in the active site, which mimics a glycosyl-enzyme intermediate at the transition state. Therefore, human FucT V is believed to employ an ordered sequential mechanism with donor GDP-Fuc binding first, followed by acceptor binding, with the product Le^x being released first followed by the GDP portion of the donor (Qiao *et al.*, 1996). For human FucT V, both GTP and GDP are potent inhibitors, whereas the inhibitory effect of GMP is moderate and guanosine is not an inhibitor (Murray *et al.*, 1996). This suggests that both the α- and β-phosphate groups of GDP-Fuc are involved in binding to the enzyme-bound intermediate. The secondary isotope effect and inhibitory pattern of GDP-2F-fucose on the human V-catalyzed reaction supports the hypothesis that glycosidic bond cleavage (C1-O1) occurs prior to the nucleophilic attack on the anomeric position of GDP-Fuc (Murray *et al.*, 1997).

1.4.3 Plant α1,4 FucTs

It has been observed that α1,3/4 FucTs with a significant level of α1,4 activity seem to be widely present in many plants, including mungbean (Crawley *et al.*, 1989), sycamore (Fitchette-Laine *et al.*, 1997) and *Vaccinus myrtillus* (Melo *et al.*, 1997). The cDNAs that are predicted to encode α1,3/4 FucTs with a significant level of α1,4 activity have also cloned from *Beta vulgaris* (Bakker *et al.*, 2001), *Lycopersicum aesculentum* (Wilson, 2001), *Arabidopsis thaliana* (Leonard *et al.*, 2002) and *Silene alba* (Leonard *et al.*, 2005). α1,4 FucT from *A. thaliana* was found to contain the conserved Trp residue (Bakker *et al.*, 2001; Leonard *et al.*, 2002), which was identified in human FucT III as conferring the Type I acceptor specificity (Dupuy *et al.*, 1999; Dupuy *et al.*, 2004).

1.4.4 *H. pylori* FucTs

1.4.4.1 *H. pylori fut* Genes

The *H. pylori* genome sequence contains two paralogous genes *futA* and *futB* that encode two α1,3/4 FucTs, FucTa and FucTb, respectively. FucTa from strain NCTC11639 and FucTb from strain NCTC11637 were first characterized in 1997. Similar to human FucT IV, both enzymes were reported to have α1,3 activity exclusively (Ge *et al.*, 1997; Martin *et al.*, 1997). In contrast, FucTa from strain UA948 was subsequently cloned and characterized, and like human FucT V, it possesses both α1,3 and α1,4 activities at substantial levels, but with a Type II acceptor preference (Rasko *et al.*, 2000b). Later, FucTa and FucTb proteins from strain UA1111 were characterized and both displayed very low levels of α1,3 and α1,4 activity (Rasko *et al.*, 2000a). A recent study reported that a *H. pylori* α1,3/4 FucT from strain DMS6709 possesses primarily α1,4 activity but little α1,3 activity (Rabbani *et al.*, 2005), very similar to human FucT III (Table 1-4). FucTa proteins from strains NCTC11639 and UA948 have been cloned and examined in our laboratory (Ge *et al.*, 1997; Rasko *et al.*, 2000b), therefore they were chosen in the current study for detailed characterization.

The gene encoding α1,2 FucT has been named *futC* (Berg *et al.*, 1997). The function of *H. pylori* α1,2FucT was first characterized in *H. pylori* strains UA802 and 26695. When *futC* was knocked out, the Le^y structure was no longer detected by enzyme-linked immunosorbent assay (ELISA) or immunoelectron microscopy (Wang *et al.*, 1999b).

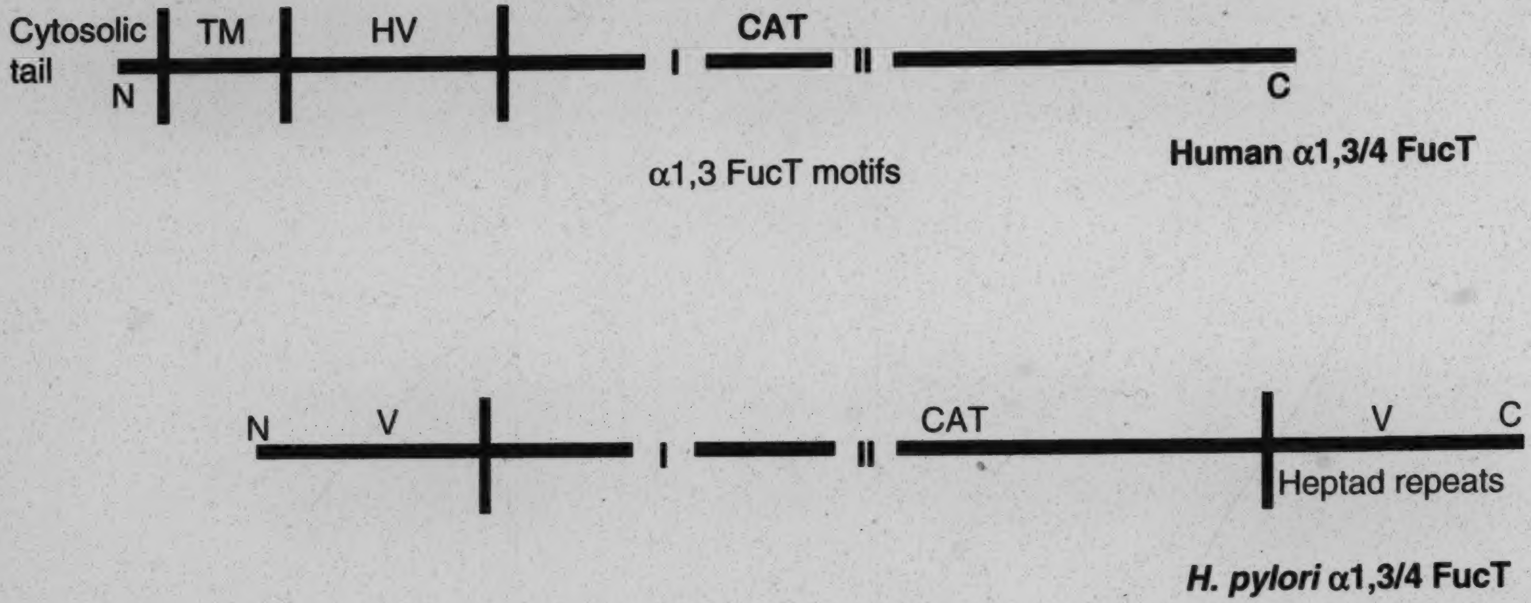
1.4.4.2 α1,3/4 FucTs

H. pylori α1,3/4 FucTs from different strains share more than 70% sequence identity. The internal catalytic domain is highly conserved whereas the N- and C- termini are divergent. Alignment of the *H. pylori* α1,3/4 FucTs with mammalian counterparts demonstrates very low sequence homology, which is restricted to the two α1,3/4 FucT motifs within the catalytic domain (Ge *et al.*, 1997; Martin *et al.*, 1997) (Fig. 1-9). In comparison with mammalian α1,3/4 FucTs, *H. pylori* enzymes do not have an N-terminal tail or transmembrane domain, but they contain a heptad repeat region at their C-terminus that is absent in mammalian α1,3/4 FucTs (Ge *et al.*, 1997; Ma *et al.*, 2003; Martin *et al.*, 1997; Rasko *et al.*, 2000b). It was suggested that this heptad repeat region contains a

Table 1-4. α1,3 and α1,4 activities of *H. pylori* α1,3/4 fucosyltransfeases.

FucT	α1,3 activity	α1,4 activity	Reference
11639FucTa	High	No activity detected	(Ge *et al.*, 1997)
11637FucTb	High	No activity detected	(Martin *et al.*, 1997)
UA948FucTa	High	High	(Rasko *et al.*, 2000b)
1111FucTa	Low	Low	(Rasko *et al.*, 2000a)
1111FucTa	Low	Low	(Rasko *et al.*, 2000a)
DMS6709FucT	Low	High	(Rabbani *et al.*, 2005)

Fig 1-9. Comparison of the domain construction of human and *H. pylori* α1,3/4 fucosyltransferases (FucTs). N: N-terminus; C: C-terminus; TM: transmembrane domain; HV: hypervariable region (stem region); CAT: catalytic domain; V: variable region. Adapted from (Rasko *et al.*, 2000b).


Cytosolic tail
TM
HV
CAT
N
I
II
C
α1,3 FucT motifs
Human α1,3/4 FucT
N
V
I
II
CAT
V
C
Heptad repeats
H. pylori α1,3/4 FucT

leucine-zipper-like motif mediating dimer formation (Ge *et al.*, 1997; Martin *et al.*, 1997). Nevertheless, supportive experimental evidence remains to be obtained.

1.4.4.3 α1,2 FucTs

H. pylori α1,2FucTs from different *H. pylori* strains share very high sequence identity (~95%). But when compared to their mammalian and bacterial counterparts, very low identity 18%, and 22%, respectively is obtained (Oriol *et al.*, 1999). *H. pylori* α1,2 FucTs also lack the N-terminal cytosolic tail and the transmembrane domain. In fact, the α1,2 FucT from *H. pylori* strain UA802 is reported to be a soluble protein located in the cytoplasm (Wang *et al.*, 1999a).

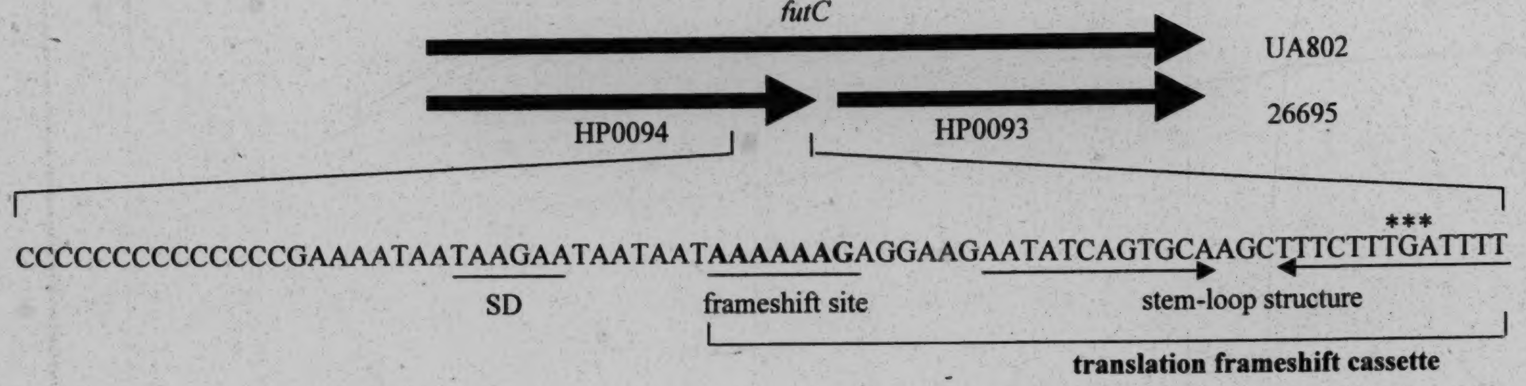
1.4.4.4 Phase Variation

The *futA, futB* and *futC* genes from different *H. pylori* strains do not always encode functional FucT proteins because these genes contain a polyA-polyC tract near the 5' end. During DNA replication, the addition or deletion of one or more base pairs within the tracts occurs at a much higher rate than the normal mutation frequency. This leads to the on/off status of the *fut* gene at both transcriptional and translational levels (Appelmelk *et al.*, 1998; Appelmelk *et al.*, 1999; Appelmelk *et al.*, 2000; Wang *et al.*, 1999a; Wang *et al.*, 1999b; Wang *et al.*, 2000). This phenomenon is called phase variation, a very common mechanism used by microorganisms to switch on or off the expression of outer membrane proteins (Bart *et al.*, 1999; Horino *et al.*, 2003; Kyme *et al.*, 2003; Martin *et al.*, 2005; Neyrolles *et al.*, 1999; Owen *et al.*, 1996; Seifert, 1996; Zhang *et al.*, 2004). Phase variation caused by mutation in the polyA or polyC tract has been experimentally observed in *futA* in strains J99 and NCTC11639 (Wang *et al.*, 2000), and in *futB* in strain NCTC11637 (Appelmelk *et al.*, 1999; Wang *et al.*, 1999b). The rate of phase variation resulting in the on/off status of Lewis antigen expression in *H. pylori* is approximately 0.2-0.5% (Appelmelk *et al.*, 1998; Appelmelk *et al.*, 1999; Appelmelk *et al.*, 2000).

In addition to the presence of the short repeat sequence (polyA-polyC tract), *futC* contains imperfect TAA (or GAA or AAA) repeats immediately downstream of the poly C tract at the mid-region of the gene (Wang *et al.*, 1999b). The signature sequences, an internal Shine-Dalgarno (SD)-like context, a heptamer (AAAAAAG) and the downstream potential stem-loop structure, are also present and they are hypermutable (Wang *et al.*, 1999b). During translation, the ribosome can slip to the –1 reading frame at a frequency of 50%. The presence of the SD-like context and the stem-loop structure can enhance the slippage of frameshift by interacting with the ribosomal components (Fig. 1-10). Such a mechanism has been confirmed in strain 26695, whose *futC* gene contains two truncated reading frames (HP0094 and HP0093) but which are able to switch on as a single open reading frame to encode the full-length α1,2 FucT, resulting in Le^y expression on the LPS structure (Wang *et al.*, 1999b).

In addition to the polyA-polyC slippery tracts and the signature sequences of a –1 ribosomal frameshift to switch the *fut* gene on or off, other mechanisms are also present accounting for the off-status of *fut* genes. For instance, *futC* in *H. pylori* strain 1174 has the insertion of 2C and 2A at the hypermutable region generating a frameshift mutation. But this gene lacks a translation frameshift cassette (AAAAAAG) so the frameshift cannot be compensated, giving rise to a constant off status of *futC* (Wang *et al.*, 1999b). The *futC* gene in strain UA1218 has 80 nucleotides deleted at its promoter region (Wang *et al.*, 1999b) so that it fails to be properly translated. The mutations outside of the

Fig. 1-10. The *futC* gene from strains UA802 and 26695 encoding α1,2 FucTs. Thick arrows represent the orientation and extent of the open reading frames of *futC*, below which the nucleotide sequences of putative 26695 α1,2 *futC* translation frameshift cassette is given. Shine-Dalgarno (SD) indicates an internal Shine-Dalgarno-like sequence. The AAAAAAG heptamer (bold) is a highly slippery sequence. The two inverted arrows represent the sequences that potentially form a stem-loop structure. The TGA labeled with asterisks corresponds to the stop codon of HP0094. Both the SD sequence and the stem-loop structure can enhance the −1 frameshift of the ribosome at AAAAAAG site. From (Wang *et al.*, 1999b) with permission from the corresponding author.


futC
UA802
HP0094
HP0093
26695
***
CCCCCCCCCCCCCGAAAATAATTAAGAATAATAATAAAAAAGAGGAAGAATATCAGTGCAAGCTTTCTTTGATTTT
SD
frameshift site
stem-loop structure
translation frameshift cassette

polyA-polyC region of *futB* from strain UA948 account for the persistent off-status of the gene (Rasko *et al.*, 2000b).

Notably, the α1,3/4FucTs from the majority of *H. pylori* strains possess much higher activity than the α1,2FucT in the same strain (Wang *et al.*, 1999a; Wang *et al.*, 1999b; Wang *et al.*, 2000). This might explain the observation that *H. pylori* LPS generally contains the polymeric Le^x but only a monomeric Le^y at the terminal end of O-antigen. To predict the Lewis antigen expression pattern in a particular *H. pylori* strain, one needs to consider the on/off status of the *futA, futB and futC* genes, the activity and the expression levels of the functional α1,3/4FucTs and α1,2FucTs, and preferences of these enzymes to utilize distinctive substrate structures.

1.4.5 Predicted FucT Fold

Combining HCA and fold recognition methods, Breton *et al.* proposed that α1,2 and α1,3 FucTs likely share the GT-B fold with β-glucosyltransferase from phage T4 (BGT) – two domains consisting of an α/β/α structure with a wide cleft in between where the donor and acceptor bind (Breton *et al.*, 1996). Based on PSI-BLAST search and structural analysis, α1,3/4 FucTs (CAZY family 10) are also predicted to adopt the GT-B fold (Liu and Mushegian, 2003; Wrabl and Grishin, 2001). In contrast, results from threading analysis argues against the FucT VII adopting the GT-B fold (de Vries *et al.*, 2001c), instead it was predicted that FucT III and VII adopt the barrel fold of tryptophan synthase from *Salmonella typhimurium* (de Vries *et al.*, 2001a). α1,2 FucTs (CAZY family 11) and α1,6 FucTs (CAZY family 23) are grouped into the unclassified GT-D fold family (Kikuchi *et al.*, 2003). Currently there are no crystal structures resolved for any member of the FucT family and virtually no sequence homology is shared between FucTs with other GT families. This makes modeling studies particularly difficult.

1.5 Carbohydrate-based Therapeutics and Application of GTs

Fucosylated carbohydrate structures, especially sialyl-Le^x and silayl-Le^a as the ligands for E-selectin, P-selectin and L-selecin (Kannagi, 2004), are central to cell-cell interactions and cell migration in association with physiological and pathological processes, such as recruitment of leukocytes to sites of infection and the homing of leukocytes to lymph nodes (Lowe, 2001; Vestweber and Blanks, 1999), embryogenesis (Eggens *et al.*, 1989), neural development (Marani and Mai, 1992; Streit *et al.*, 1996), angiogenesis (Nguyen *et al.*, 1993), fertilization (Johnston *et al.*, 1998; Oehninger *et al.*, 1998), tumor metastasis (Kannagi, 2004 ; Magnani, 2004), and bacterial adhesion (Ilver *et al.*, 1998) and infection (Herron *et al.*, 2000). It is worth emphasizing that sialyl-Le^x and sialy-Le^a are highly expressed in many malignant cancers and their expression is closely correlated with the status of hematogenous metastasis and tumor angiogenesis, thus reflecting the malignant nature of cancer cells. Hence sialyl-Le^x and sialyl-Le^a are two carbohydrate tumor markers for cancer diagnosis and prognosis (Kannagi, 2004). As such, inhibition of binding of sialyl-Le$^{x/a}$ to the selectins becomes a potential target for treatment of cancer and inflammatory diseases. Several strategies have been proposed and investigated: antibodies to sialyl-Le$^{x/a}$ (Hosono *et al.*, 1998; Nakashio *et al.*, 1997), selectin inhibitors (Abraham *et al.*, 1999; Huang *et al.*, 2000; Romano and Slee, 2001; Schon *et al.*, 2002; Simanek *et al.*, 1998) including small molecules that mimic the structures of sialyl- Le$^{x/a}$ determinant (Huang *et al.*, 1999; O *et al.*, 2002; Romano, 2005), and anti-sense-cDNA of the genes for the synthetic enzymes (FucT IV, FucT VII) (Hiraiwa *et al.*, 1996; Nishiwaki *et al.*, 2003; Weston *et al.*, 1999).

With recent significant strides in glycobiology and glycomics, carbohydrate-based drug development has been recognized as a promising field in the pharmaceutical industry. Anti-adhesion treatment using oligosaccharides in bacterial and viral infections is expected to efficiently block the first step of infection (Kobata, 2003). Carbohydrate-based vaccines have been exploited in bacterial, viral and parasite infections (Goldblatt, 1998) and cancers (Barchi, 2000; Danishefsky and Allen, 2000). Meanwhile, inhibitors of GTs can also be an alternative strategy for treatment of many diseases caused by impaired glycosylation (Compain and Martin, 2001). α1,3 FucT inhibitors, for instance, could be developed to control arthritis (Springer and Lasky, 1991) and tumor growth (Osborn, 1990). The xenotransplant rejection caused by the recipient producing anti-Gal antibodies could be overcome using α1,3 galactosyltransferase inhibitors (Chen *et al.*, 1999b; Wang *et al.*, 1997b). Therefore, in-depth understanding of the function and mechanisms of GTs is essential for the rational design of such novel drugs. Moreover, GTs could be utilized to synthesize carbohydrate-based vaccines, glycomimetics, and antibiotics that contain sugar structures (Barrett *et al.*, 2004; Garneau *et al.*, 2004; Kruger *et al.*, 2005; Losey *et al.*, 2001; Losey *et al.*, 2002; Walsh *et al.*, 2003a; Walsh *et al.*, 2003b). When GTs are combined with other biological catalysts, complex glycoconjugates can also be efficiently produced (Franke *et al.*, 2003; Koeller and Wong, 2000; Mong *et al.*, 2003; Mong and Wong; 2002).

Enzymatic synthetic pathways, with the advantages of being highly stereo- and regioselective and with almost no side products formed in reaction, is more favorable in the large-scale production of oligosaccharides than chemical synthetic pathways, which needs multiple protection and deprotection steps (Endo and Koizumi, 2000; Ichikawa *et al.*, 1992). Nevertheless, preparation of sugar nucleotides using traditional chemical synthetic methods is laborious and costly. The preparation of large quantities of soluble and stable GTs had also been a serious problem. Recent progress in genetic engineering has made GTs available for large-scale production of carbohydrates (Blixt *et al.*, 2005; Dumon *et al.*, 2004; Fabre *et al.*, 2005; Hoshi *et al.*, 2004; Kamath *et al.*, 2004; Priem *et al.*, 2002; Yamamoto *et al.*, 1998). In addition, employing protocols for the regeneration of sugar nucleotides in conjunction with GT catalysis dramatically facilitates the production of sugar nucleotides (Elling *et al.*, 1993; Koeller and Wong, 2000 and references therein). In addition, the recent development of one-pot programmable synthesis of oligosaccharides using coupled multienzyme systems (Bulter and Elling, 1999; Bulter *et al.*, 2001; Hokke *et al.*, 1996; Lee *et al.*, 2004; Ma and Stockigt, 2001; Oh *et al.*, 2003; Yu *et al.*, 2004) and the automatic solid-phase synthetic technology in oligosaccharide synthesis have remarkably revolutionized synthetic chemistry.

In short, characterizing the structure-function relationships, catalytic mechanisms and crystal structures of GTs would facilitate the effective utilization of these enzymes in new drug development and glycoconjugate synthesis. The development of carbohydrate microarray or GlycoChip® (http://www.glycochip.com) allows high-throughput screening of the carbohydrate-protein interactions (Feizi *et al.*, 2003) and facilitates screening new glycomimetics and inhibitors for protein-glycan interactions (Hirabayashi, 2003). Such technology will expedite the study of GTs; and conversely, GTs could be used to synthesize various oligosaccharide probes for the array.

1.6 Goals and Significance of Project

Despite the extensive characterization of mammalian α1,3/4 FucTs, the study of the prokaryotic FucTs is still in its infancy. As *H. pylori* α1,3/4FucTs share very low sequence similarity to their mammalian counterparts, it might be expected that these bacterial α1,3/4 FucTs possess some unique features. My project was to investigate the structure-function relationship of *H. pylori* α1,3/4 FucTs and thus to elucidate their mechanisms. The functional characterization of *H. pylori* α1,3/4 FucTs included five aspects: 1. Identification of the domain determining Type I specificity in *H. pylori* α1,3/4 FucTs (Chapter 2); 2. Determination of the amino acid(s) that control(s) Type I acceptor recognition in *H. pylori* α1,3/4 FucTs (Chapter 3); 3. Delineation of the minimal catalytic domain of *H. pylori* α1,3/4 FucTs and purification of soluble, active and stable enzyme for detailed kinetic analysis (Chapter 4); 4. Mapping the key polar groups in Type II and Type I acceptor for recognition by *H. pylori* α1,3/4 FucTs (Chapter 4) and 5. Exploring the potential of utilizing *H. pylori* α1,3/4 FucTs in Le^x and Le^a synthesis at milligram scale (Chapter 4).

The data in this thesis demonstrate that despite the low sequence homology *H. pylori* α1,3/4 FucTs share remarkable functional similarities with their mammalian counterparts. The current study thus greatly advances our understanding of the prokaryotic FucTs and expands the current literature. Since bacterial enzymes are relatively easy to work with as compared to mammalian enzymes, *H. pylori* α1,3/4 FucTs become an attractive model system for studying the entire FucT family. In addition, structure-function characterization of *H. pylori* FucTs would facilitate the modification of the Lewis antigen expression patterns by mutating FucT genes, thereby enabling the design of experiments to elucidate the role of Lewis antigen in *H. pylori* pathogenesis. These studies would also assist in engineering novel enzymes with improved activity and specificity to facilitate chemoenzymatic synthesis of glycoconjugates.

Carboxyl Terminal Amino Acids of *Helicobacter pylori* α1,3/4 Fucosyltransferases Determine Type I and Type II Transfer

A version of this work has been published previously[1]:

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[1]: All experiments were performed by Ma B.

2.1 Introduction

H. pylori LPS contains fucosylated oligosaccharides, predominantly the Type II blood group antigens Le^x and Le^y (Wirth *et al.*, 1996). Some *H. pylori* strains also express the Type I blood group antigens Le^a and Le^b (Monteiro *et al.*, 1998a). *H. pylori* FucTs are the enzymes responsible for the last steps in the synthesis of Lewis antigens. Difucosylated Lewis antigens (Le^y and Le^b) can be synthesized via two pathways: terminal fucosylation (in α1,2 linkage) followed by subterminal fucosylation (in α1,3 or α1,4 linkage) or subterminal fucosylation followed by terminal fucosylation. Unlike mammalian cells, the latter pathway is predominately used in *H. pylori* (Wang *et al.*, 2000).

In *H. pylori* genomes (Alm *et al.*, 1999; Tomb *et al.*, 1997), there exist two homologous α1,3/4 FucT genes, *futA* and *futB* and one gene *futC* for α1,2 FucT (Alm *et al.*, 1999; Tomb *et al.*, 1997; Wang *et al.*, 1999b). These three *fut* genes do not always encode functional proteins. For instance, the *futA* gene, but not the *futB* gene, encodes an active α1,3/4 FucT in *H. pylori* strains NCTC11639 and UA948 (Ge *et al.*, 1997; Rasko *et al.*, 2000b). The *futC* gene in strain NCTC11639 is functional (Wang *et al.*, 1999b), but this gene is not functional in strain UA948 (Rasko *et al.*, 2000b). The on/off status of *fut* genes and the various levels of FucT activities exhibited in different *H. pylori* strains determine the Lewis antigen expression patterns of *H. pylori* LPS.

α1,2, α1,3/4 and α1,6 FucTs are inverting enzymes and belong to GT family 11, 10, 23 respectively (Campbell *et al.*, 1997). They are widely expressed in vertebrates, invertebrates and bacteria. Mammalian α1,3/4 FucTs are Golgi-anchored proteins with a short cytoplasmic N-terminal tail, a transmembrane segment, hypervariable stem domain and C-terminal catalytic domain. The C-terminal catalytic domain of mammalian FucTs displays a low level of amino acid sequence identity (40%) with the internal catalytic domain of *H. pylori* FucTs (Rasko *et al.*, 2000b). This domain contains two highly conserved regions, referred to as the α1,3/4 FucT motifs (Breton *et al.*, 1996, 1998). In comparison with mammalian FucTs, *H. pylori* FucTs do not have N-terminal tails or transmembrane domains, instead, they contain heptad repeats at their C-terminus, which are absent in human FucTs (Ge *et al.*, 1997; Rasko *et al.*, 2000b).

α1,3/4 FucTs exhibit distinct acceptor preferences. For instance, despite the fact that human FucT III, V, VI and bovine FucT share at least 70% protein sequence identity (Oulmouden *et al.*, 1997; Weston *et al.*, 1992), they displayed strikingly distinctive acceptor specificity patterns. FucT VI and bovine FucT display exclusive α1,3 activity, in contrast, FucT III and V show both α1,3 and α1,4 activity, although they exhibit different preferences towards Type I and Type II substrates. FucT III favors Type I acceptors therefore it is predominantly an α1,4 FucT, whereas FucT V prefers Type II acceptors hence it is primarily an α1,3 FucT (de Vries *et al.*, 2001a and reference therein; Oulmouden *et al.*, 1997; Weston *et al.*, 1992). The domain swapping experiments performed between FucT III and VI (Legault *et al.*, 1995) or between FucT III and V (Xu *et al.*, 1996) demonstrated that the N-terminal hypervariable stem region in human FucTs is responsible for conferring acceptor specificity. In particular, site-directed mutagenesis data showed that the substitution of as few as two amino acids (His^{87}Ile^{88} in FucT III corresponding to Asn^{86}Thr^{87}in FucT V) (Nguyen *et al.*, 1998) or replacement of a single amino acid (Trp^{111} in FucT V corresponding to Arg^{115} in bovine FucT) (Dupuy *et al.*, 1999), is able to switch the Type I or Type II substrate preference. The adjacent residue Asp^{112} in FucT V, corresponding to Glu^{116} in bovine FucT, was able to further modulate the relative Type I and Type II acceptor specificity (Dupuy *et al.*, 1999). Since there is no sequence identity at the N-terminus between human FucTs and *H. pylori* FucTs (Rasko *et al.*, 2000b), no domain or amino acid residues in *H. pylori* FucTs correspond to the region or residues conferring Type I and Type II substrate specificity in human FucTs.

The main goal of this study was to identify the determinants of *H. pylori* FucTs responsible for discriminating acceptor substrate preference. Characterization of structure-function relationships of *H. pylori* FucTs would enable us to manipulate the Lewis antigen expression pattern by mutation of FucT genes. This could lead to a clearer understanding of the role of Lewis antigen in persistent *H. pylori* infection. In addition, *H. pylori* FucTs are promising candidates for chemoenzymatic glycoconjugate synthesis (Ernst and Oehrlein, 1999; Johnston *et al.*, 1998; Meldal and St Hilaire, 1997). Finally, better understanding of the bacterial FucTs could help us understand the divergent evolution of various lineages in FucT kingdom.

2.2. MATERIALS AND EXPERIMENTAL PROCEDURES

2.2.1 Materials

Primers were synthesized by Invitrogen Canada Co. (Burlington, Ontario). pGEM-T vector was obtained from Promega Co. (Madison, WI). Type II-R (βGal1-4βGlcNAc-O-$(CH_2)_8CO_2CH_3$) and Type I-R (βGal1-3βGlcNAc-O-$(CH_2)_8CO_2CH_3$) were kindly provided by Dr. O. Hindsgaul. Anti-penta-histidine monoclonal antibody was purchased from QIAGEN Co. (Mississauga, ON). GDP-fucose and horse-radish peroxidase conjugated goat anti-mouse IgG were from Sigma Chemical Co. (St Louis, MO). GDP-^{3}[H]Fuc (0.1 mCi ml^{-1}, 17.3 Ci mmol^{-1}) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). BCA protein assay kit was purchased from Pierce

Biotech Inc. (Rockford, IL). Nitrocellulose membrane was obtained from Micron Separation Inc. (Westboro, MA). Enhanced chemiluminescence (ECL) kit was purchased from Amersham Biosciences Corp. (Buckinghamshire, England). BioMax MR film was obtained from Eastman Kodak Co. (Rochester, NY).

2.2.2 Cloning of Chimeric FucTs

H. pylori strains NCTC11639 and UA948 were cultured by standard methods, as described by Taylor *et al.* (Taylor *et al.*, 1987). Chromosomal DNA was isolated following standard protocol (Sambrook and Russel, 2001). Two primers BM11 and BM12 were designed to amplify the *futA* gene (Table 2-1). The domain swapping experiments were accomplished by primer overlap extension with two steps of sequential PCR (Sambrook and Russel, 2001). The sequences of primers are given in Table 2-1. Two pairs of proximal primers were used in the second step of PCR, BM91 and BM77, complementary to *futA* in strain 11639, and BM101 and BM87, complementary to *futA* in strain UA948. A Shine-Dalgarno sequence was included in the sense primers BM91 and BM101. The codons for six histidine residues were included in the antisense primers BM77 and BM87. The PCR products were cloned into A/T cloning vector pGEM-T under the control of a T7 promoter. Cloned chimeric *futA* genes were sequenced to assure no mutation had occurred during their construction.

2.2.3 Induction and Expression of *H. pylori* FucT Genes

Expression of FucT genes was induced as described previously (Ge *et al.*, 1997; Rasko *et al.*, 2000b) with minor modifications. *E. coli* HMS174DE3 cells were used for FucT expression, DE3 carries the gene encoding T7 RNA polymerase under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-induced lactose promoter. Cells were

Table 2-1. Sequences of primers used in domain swapping experiments. [a]*EcoR*I and *Kpn*I restriction enzyme sites are shown in lower case (The A/T cloning vector pGEM-T was used for cloning in the current study, so the enzyme recognition site was not used). The linker region is in italic. Codons coding for six histidine residues were included at the 3' end of antisense primers BM77 and BM87, and are underlined. A Shine-Dalgarno sequence is included in sense primers BM91 and BM101 and is in bold italic. The start and stop codon are in bold. [b]NA: not applicable.

Primers	Sequences	Position in 11639 *futA*	Position in UA948 *futA*
BM11	5' GCG ATA CCT TTT GCT CTT GTG 3' (sense)	-58 — -78	-58 — -78
BM12	5' TGG AGT TTT GAA GTG GTG GAT G 3' (antisense)	-58 — -78	-58 — -78
BM3	5' GAT TAC GCC ATA GGC TTT G 3' (sense)	313—331	313—331
BM4	5' CAA AGC CTA TGG CGT AAT C 3' (antisense)	313—331	313—331
BM7	5' CTT TAA AAA AAC CTT CCC AT 3' (sense)	455—475	449—468
BM8	5' ATG GGA AGG TTT TTT TAA AG 3' (antisense)	455—475	449—468
BM5	5' CCT ATT TAT TGG GGG AGT C 3' (sense)	781—799	775—793
BM6	5' GAC TCC CCC AAT AAA TAG G 3' (antisense)	781—799	775—793
BM9	5' TCG TGA TTT GAA TGA GCC 3' (sense)	1059—1176	1062—1079
BM10	5' GGC TCA TTC AAA TCA CGA 3' (antisense)	1059—1176	1062—1079
BM51	5' CGA TAA CCC TTT CAT TTT CTG TCG TGA TTT GAA TGA GCC 3' (sense)	1038—1076	NA[b]
BM52	5' CAG AAA ATG AAA GGG TTA TCG TGA TAA ATC GTA TCG TTT 3' (antisense)	1020—1058	NA[b]
BM61	5' GCA ATG ATG CCC ATT ATT CTG CTC TTC ATC GTG ATT TGA ATG AG 3' (sense)	NA[b]	1034—1077
BM62	5' AAG AGC AGA ATA ATG GGC ATC ATT GCA ATG ATA AAT CGT ATC GT 3' (antisense)	NA[b]	1016—1059
BM91[a]	5' *ATA T*ga att ***cTA AGG AGG TCT AAA*** **ATG** TTC CAA CCC CTA TTA GAC GCT 3' (sense)	1—24	1—24
BM77[a]	5' *TAT* agg gac c**TT** AGT GAT GGT GAT GGT GAT G*TG* CCA AAC CCA ATT TTT TAA CCC A 3' (antisense)	1414—1437	NA[b]
BM101[a]	5' *ATA T*ga att ***cTA AGG AGG TCT AAA*** **ATG** TTC CAG CCC TTA CTA GAC GCT 3' (sense)	1—24	1—24
BM87[a]	5' *TAT* agg tac c**CT** AGT GAT GGT GAT GGT GAT G*TG C*TT TTC TAA CCC ACC TCC 3' (antisense)	NA[b]	1370—1389

grown at 30°C with vigorous shaking (200 rpm min^{-1}) in SOC medium (20% tryptone, 5% yeast extract, 0.05% (w/v) NaCl, 10 mM $MgCl_2$, 2.5 mM KCl, 20 mM glucose) (Sambrook and Russel, 2001) including ampicillin (100 μg ml^{-1}) and rifampicin (200 μg ml^{-1}) for 2 hours until the OD_{600} reached ~0.3 at which point 1 mM of IPTG was added. After 4 hours, the cultures were centrifuged and resuspended in HEPES buffer (20 mM, pH 7.0 containing 0.5 mM phenylmethanesulfonyl fluoride) and the cells were lysed using three freeze-thaw cycles (Wang *et al.*, 1997a). Cells were treated with lysozyme (200 μg ml^{-1}) and DNase (125 Unit ml^{-1}) for 20 min on ice after the first freeze-thaw process. The total protein concentrations of the crude cell extracts were determined using the BCA protein assay kit, with bovine serum albumin as a standard according to the supplier's instructions. The crude cell lysates (isolated membrane fractions) were used for the following enzyme assays, enzyme kinetics determination and immuno-blot analysis.

2.2.4 Fucosyltranserase Assay

FucT assays were performed as described previously (Ge *et al.*, 1997; Gosselin and Palcic, 1996; Rasko *et al.*, 2000b). A protein preparation from HMS174DE3 *E. coli* cells containing the pGEM vector was used as a negative control. The α1,3 and α1,4 FucT activities of WT and chimeric FucTs were assayed with Type II-R at 1.8 mM or Type I-R at 7.5 mM and GDP-fucose at 200 μM. GDP-^{3}[H]Fuc (~60,000 dpm, 0.2 μM) was included in each reaction. One milli unit (mU) represents the amount of enzyme that converts 1 nmol of acceptor substrate to product per minute. The specific activity (mU mg^{-1}) was obtained by dividing the enzyme activity (mU) by total protein concentration. Specific enzyme activity below 0.01 mU mg^{-1} was considered undetectable.

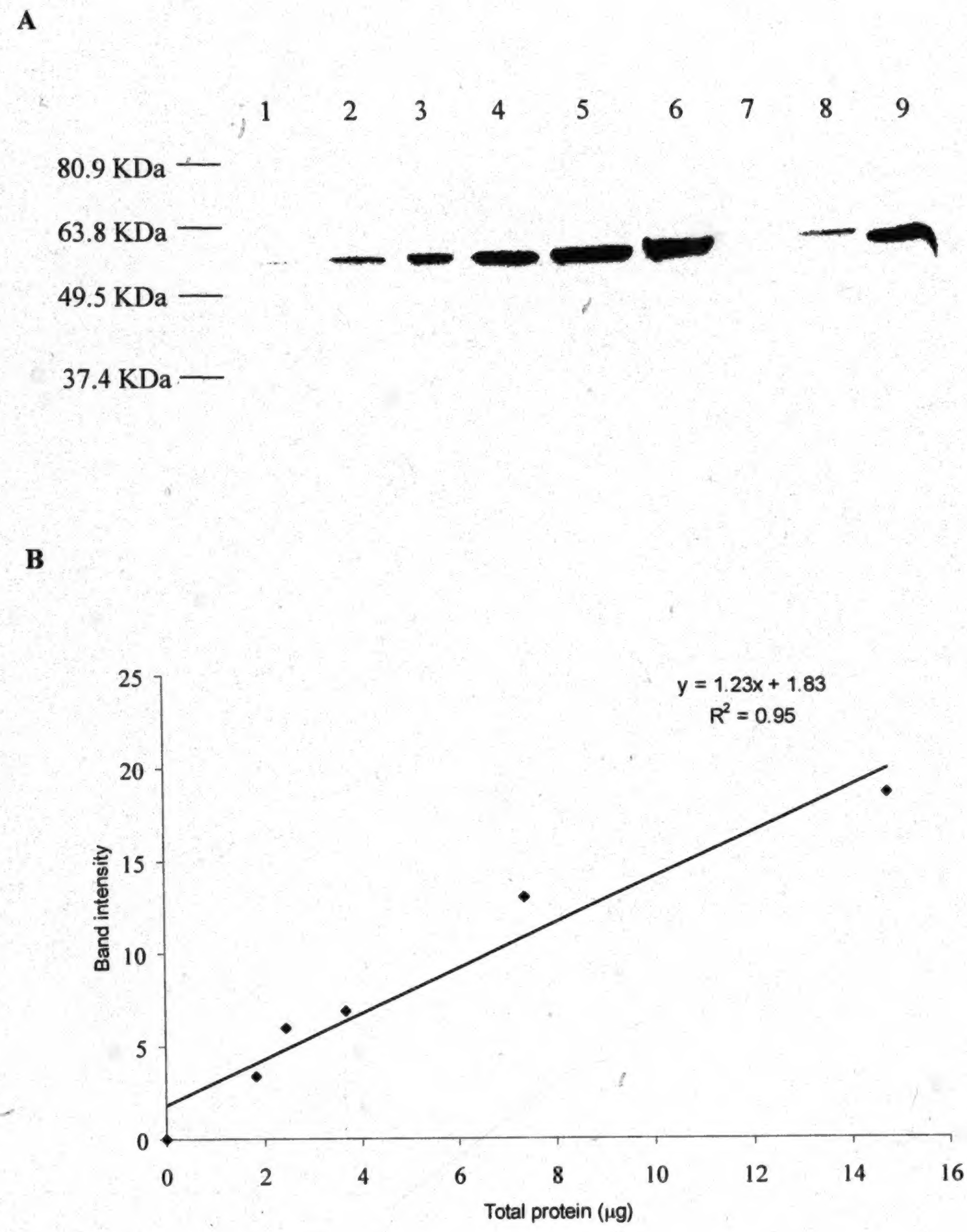
2.2.5 Immuno-Blot Analysis of Native and Chimeric FucT Expression

Crude cell extracts were boiled for 5 min in 4% (w/v) sodium dodecyl sulfate (SDS) and 0.002% (w/v) bromophenol blue. Cell extracts containing equal amounts of total protein (7.35 μg) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and the proteins were electrically transferred to nitrocellulose membrane (pore size, 0.22 μm). Nitrocellulose blots were probed with primary antibody (mouse anti-penta-histidine monoclonal antibody) at 1:1000 dilution and secondary antibody (horse-radish peroxidase conjugated goat anti-mouse IgG) at 1:2000 dilution. Blots were developed using an enhanced chemiluminescence (ECL) kit and the images were visualized on BioMax MR film. The FucT band densities were determined and quantified using Alpha Ease FC software (Alpha Innotech Coorporation, San Leandro, CA). A standard curve with six points was performed using WT UA948FucT sample containing total protein between 1.84 μg and 18.4 μg. For WT and chimeric FucT samples, 7.35 μg of total protein was loaded for immuno-blot analysis, which was in the middle of the linear range (Fig 2-1A,B). The expression level of UA948FucT was set to 1 and the expression levels of 11639FucT and chimeric FucTs were obtained relative to that of UA948FucT. The enzyme activity of mutants was normalized, by dividing the specific activity (mU mg^{-1}) by the expression level of the chimeric FucT.

2.2.6 Determination of Kinetic Parameters

Acceptor kinetics were determined using 0.03-2 mM Type II-R or 0.4-25 mM Type I-R with GDP-fucose at 200 μM including GDP-^{3}[H]Fuc. 2 mM of Type II-R is the highest concentration that can be reached in the current study due to its low solubility. Due to the limited supply of the acceptors, donor kinetics were determined using 3-200 μM GDP-fucose with Type II-R at 2 mM or Type I-R at 15 mM including GDP-^{3}[H]Fuc (0.2 μM).

Fig 2-1. Immuno-blot (A) and the standard curve (B) of FucT protein with a His_6 tag at the C-terminus. A: Wide type and chimeric FucTs with His_6-tag were expressed in *E. coli* HMS174DE3 cells. The FucT proteins were detected with mouse anti-penta-histidine monoclonal antibody (1:1000). Lane 1 – Lane 6: WT UA948FucT, crude cell extracts with total protein at 1.84 μg, 2.45 μg, 3.68 μg, 7.35 μg, 14.7 μg and 18.4 μg respectively. Lane 7: pGEM vector alone, Lane 8: $11639347^{\mathrm{CNDAHYSALH}}$, Lane 9: $11639^{1\text{-}158}\mathrm{UA948}^{157\text{-}462}$. Cell lysates in Lane 7, 8 and 9 contain 7.35 μg of total protein. **B:** Linear curve (r^2: 0.95) of immuno-blot FucT band intensity with the amount of total protein loaded in cell crude extracts. The FucT band densities were determined and quantified using Alpha Ease FC software. The linear curve shows that the band density was not saturating up to total protein at 14.7 μg.


A
1
2
3
4
5
6
7
8
9
80.9 KDa
63.8 KDa
49.5 KDa
37.4 KDa
B
y = 1.23x + 1.83
R² = 0.95
Band intensity
0
5
10
15
20
25
Total protein (µg)
0
2
4
6
8
10
12
14
16

Kinetic parameters were obtained by fitting the initial rate data to the Michaelis-Menten equation using non-linear regression analysis with Prism 2.0 software (GraphPad Software, Inc. San Diego, CA).

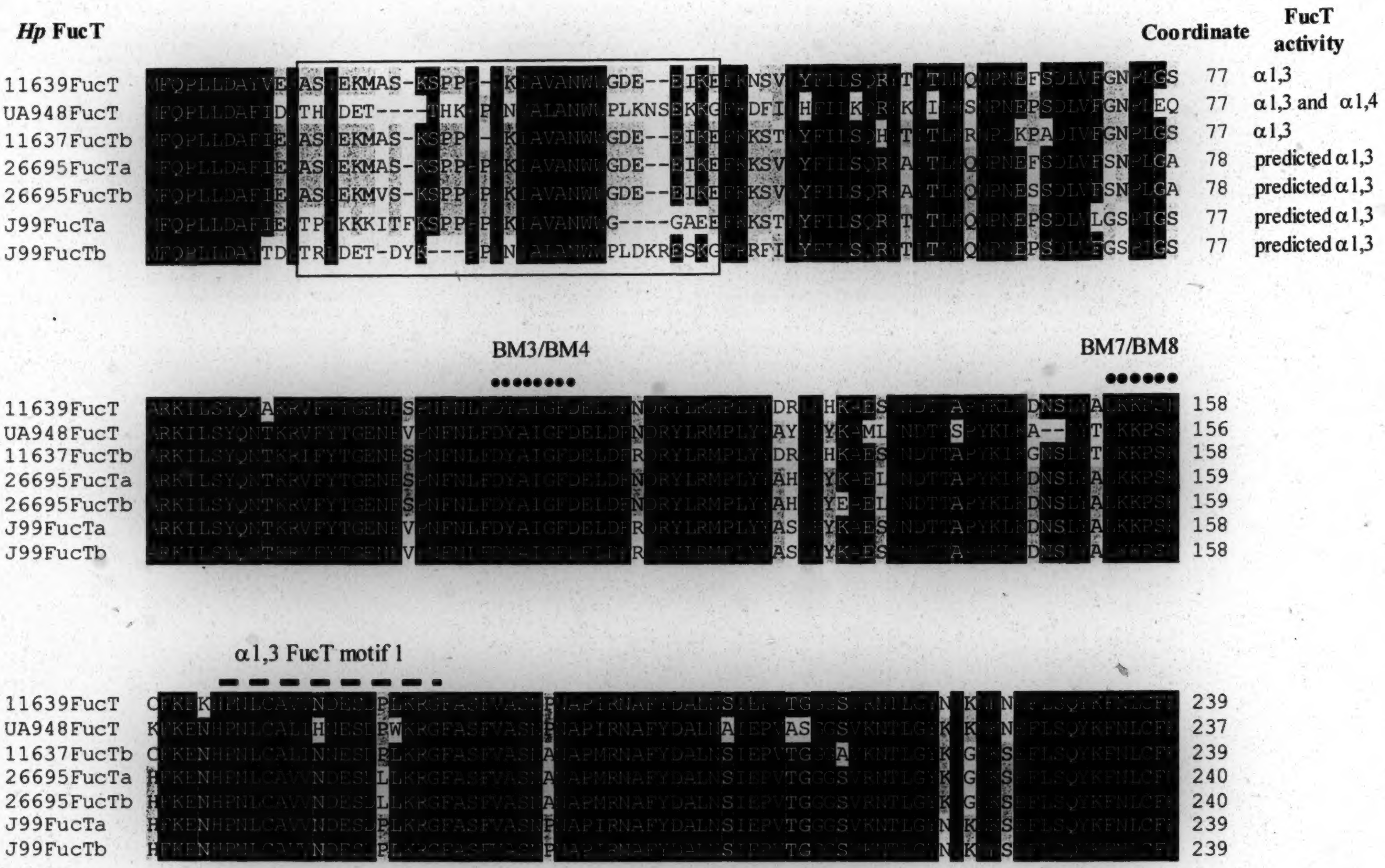
2.3 Results

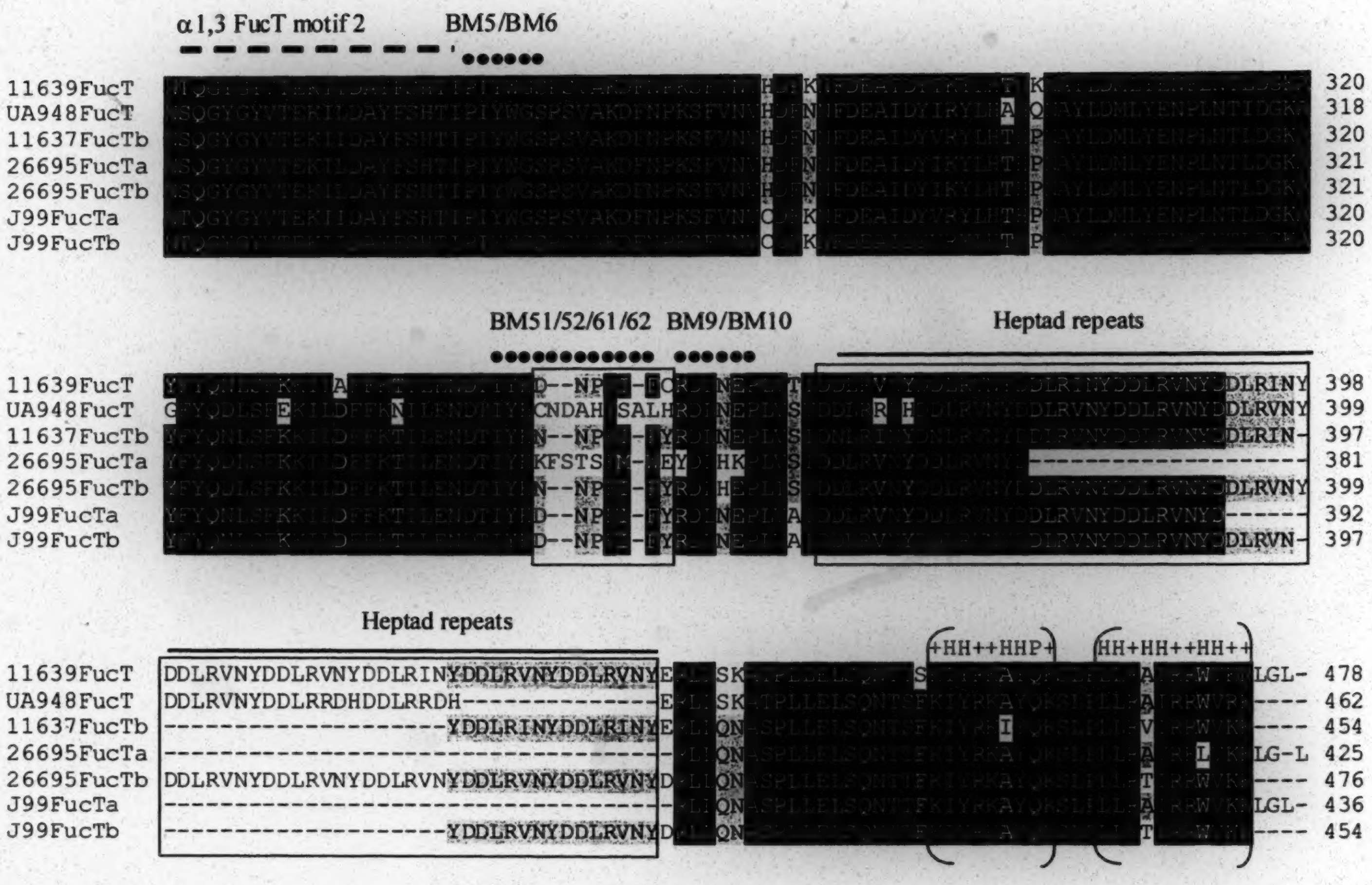
2.3.1 Sequence Alignment of *H. pylori* FucTs

Seven FucT sequences from five different *H. pylori strains* 11639, UA948, 11637, 26695 and J99 (Fig. 2-2) were aligned using ClustalW (Thompson *et al.*, 1994) with LaserGene99 software (DNAStar Inc, Madison, WI). UA948FucT displays both α1,3 and α1,4 activities (Rasko *et al.*, 2000b) and, like human FucT V, it favors Type II over Type I acceptors. 11639FucT as well as 11637FucTb have exclusive α1,3 activity (Ge *et al.*, 1997; Martin *et al.*, 1997). There is no experimental data available for the enzyme activity and specificity of 26695FucTa, 26695FucTb, J99FucTa and J99FucTb. Since *H. pylori* strain 26695 and J99 LPS express Le^x and Le^y (Appelmelk *et al.*, 1999) but not Le^a and Le^b structures, α1,3/4 FucTs in 26695 and J99 are expected to have α1,3 activity only. Whether or not the active FucT protein is encoded by the *futA* gene or the *futB* gene, or both, remains to be determined.

Three regions of low sequence identity are evident in the alignment of seven *H. pylori* FucTs (displayed in boxed areas in Fig. 2-2). The first divergent region is located at the N-terminus between residues 13 to 41 (11639FucT numbering). The second region resides immediately upstream of the heptad repeats, with most of the divergence due to the unique sequences in UA948FucT and 26695FucTa. The third divergent region contains the C-terminal heptad repeats. There are between two and ten heptad repeats with a conserved D(D/N)LR(V/I)NY sequence in all of the listed FucTs except for

Fig. 2-2. Alignment of *H. pylori* α1,3/4 FucTs from seven strains. Sequence alignment was performed using ClustalW with LaserGene99 software (DNASTAR). Highly conserved residues are in dark background, while partially conserved residues are shown in gray shaded background. Numbering at the end of each line refer to the position in the alignment. The α1,3 or α1,4 activity of each FucT is shown on the right column, and has either been determined by radiochemical assays, or predicted based on the Lewis antigen expression pattern. The positions of primers are marked with filled circles. Two highly conserved α1,3/4 motifs are marked with a dashed line. The heptad repeats are marked with a straight line. Three major divergent sequences among *H. pylori* FucTs are boxed. The sequences forming two putative amphipathic α-helices are shown in brackets, and the positively charged, hydrophobic or polar residues are marked with "+", "H" and "P" respectively.


Hp FucT
Coordinate
FucT activity
11639FucT 77 α1,3
UA948FucT 77 α1,3 and α1,4
11637FucTb 77 α1,3
26695FucTa 78 predicted α1,3
26695FucTb 78 predicted α1,3
J99FucTa 77 predicted α1,3
J99FucTb 77 predicted α1,3
BM3/BM4
BM7/BM8
11639FucT 158
UA948FucT 156
11637FucTb 158
26695FucTa 159
26695FucTb 159
J99FucTa 158
J99FucTb 158
α1,3 FucT motif 1
11639FucT 239
UA948FucT 237
11637FucTb 239
26695FucTa 240
26695FucTb 240
J99FucTa 239
J99FucTb 239


α1,3 FucT motif 2
BM5/BM6
BM51/52/61/62
BM9/BM10
Heptad repeats
Heptad repeats
+HH++HHP+
HH+HH++HH++
11639FucT
UA948FucT
11637FucTb
26695FucTa
26695FucTb
J99FucTa
J99FucTb
320
318
320
321
321
320
320
398
399
397
381
399
392
397
478
462
454
425
476
436
454

UA948FucT (Fig. 2-2). UA948FucT contains five internal repeats with this consensus sequence, while the first and the last two heptad repeats contain the amino acid sequence DDLRRDH.

2.3.2 Expression of Native and Chimeric FucTs

The expected molecular masses of 11639FucT and UA948FucT are 56.0 kDa and 54.6 kDa respectively, and both proteins migrate to the expected locations on SDS-PAGE gel (Fig. 2-3). The chimeric FucTs were expressed at similar levels as WT FucTs in HMS174DE3 cells (Fig. 2-3), indicating that the domain swapping did not cause significant changes in FucT enzyme expression levels.

2.3.3 Kinetic Parameters of WT FucTs with or without His$_6$-tag

Kinetic parameters for both WT and His$_6$-tagged WT FucTs are shown in Table 2-2. The K_m for Type II acceptor of 11639FucT is 0.37 mM, four-fold lower than that of UA948FucT. UA948FucT had a seven-fold lower K_m for Type II than Type I. 11639FucT and UA948FucT have similar donor K_m values with Type II acceptor. UA948FucT had a slightly lower donor K_m when Type I was used as an acceptor. Acceptor and donor K_m values were not affected by the addition of a His$_6$-tag, except that UA948FucT with a His$_6$-tag had a slightly higher Km for Type I acceptor.

2.3.4 Enzyme Activity and Substrate Specificity of Chimeric FucTs

Twelve chimeric FucTs (Fig 2-4A) containing peptide fragments from two parental FucTs were constructed. The exchange of the three highly divergent segments (boxed areas in Fig. 2-2) between two parental FucTs was the major focus of domain swapping. In addition, the exchange of comparatively conserved internal regions containing two α1,3 motifs was also included. The normalized α1,3 and α1,4 activities (mU mg^{-1})

Fig 2-3. Immuno-blot analysis of wild type and chimeric FucTs. Wide type and chimeric FucTs with C-terminal His$_6$-tag were expressed in *E. coli* HMS174DE3 cells with induction using 1mM of IPTG and growth at 30° for 4 h. The FucT proteins were detected with mouse anti-penta-histidine monoclonal antibody.

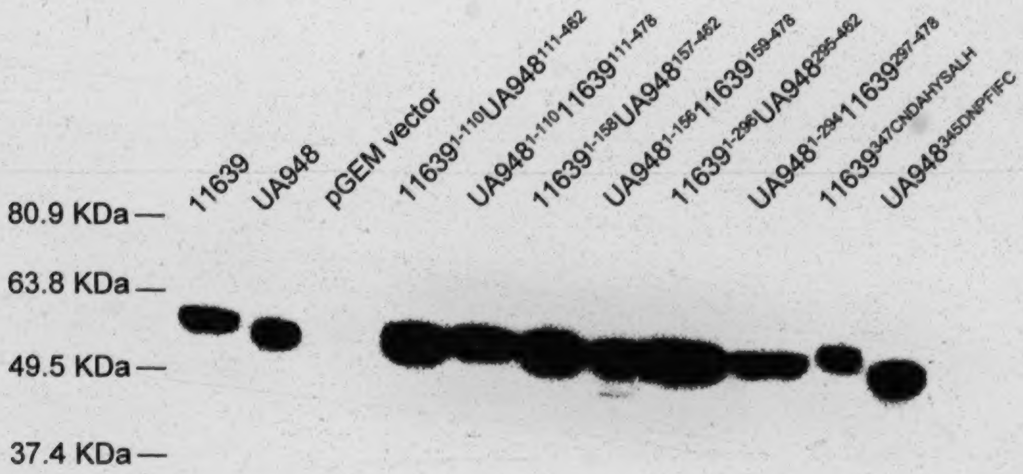

80.9 KDa
63.8 KDa
49.5 KDa
37.4 KDa
11639
UA948
pGEM vector
$11639^{1-110}UA948^{111-462}$
$UA948^{1-110}11639^{111-478}$
$11639^{1-158}UA948^{157-462}$
$UA948^{1-156}11639^{159-478}$
$11639^{1-296}UA948^{295-462}$
$UA948^{1-294}11639^{297-478}$
$11639^{347CNDAHYSALH}$
$UA948^{345DNPFIFC}$

Table 2-2. Acceptor and donor kinetic parameters of wild type 11639FucT and UA948FucT with or without the C-terminal His_6-tag. Acceptor kinetics were determined in cell crude extracts using 0.03-2 mM Type II-R, or 0.4-25 mM Type I-R with GDP-Fuc at 200 μM and GDP-^{3}[H]Fuc at 0.2 μM. Donor kinetics were determined using 3-200 μM GDP-Fuc with Type II-R at 2 mM or Type I-R at 15 mM and GDP-^{3}[H]Fuc at 0.2 μM.

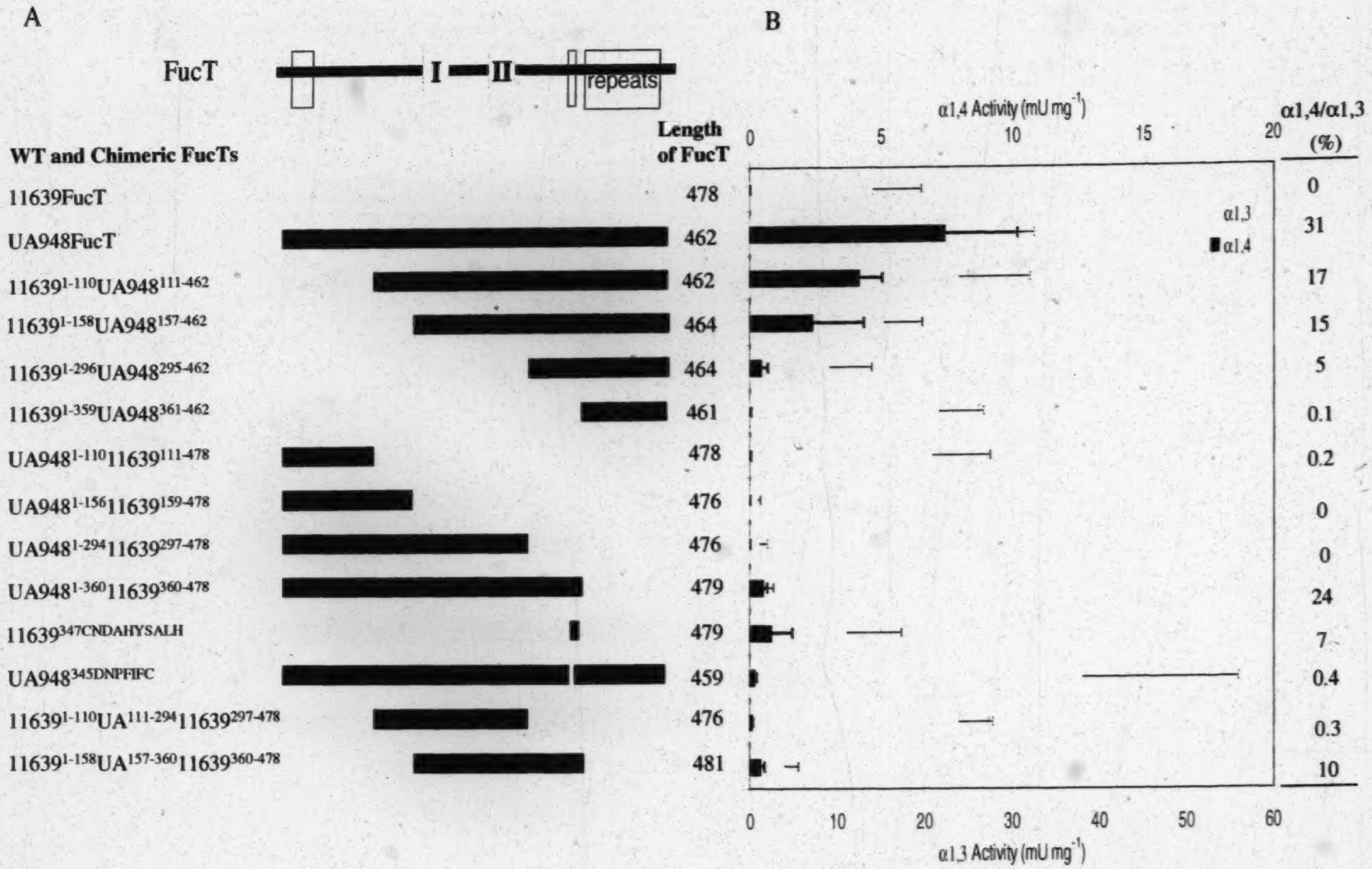
	Type II-R[a]	Type I-R[b]	K_m (μM) of GDP-fucose	
FucT constructs	K_m (mM)	K_m (mM)	with Type II-R[a]	with Type I-R[b]
11639	0.31 ± 0.06	NA[c]	48.0 ± 8.6	NA[c]
11639-C-His_6	0.37 ± 0.03	NA[c]	44.7 ± 5.5	NA[c]
UA948	1.2 ± 0.2	8.4 ± 1.1	68.3 ± 8.3	35.7 ± 7.9
UA948-C-His_6	1.5 ± 0.2	13.4 ± 1.4	56.0 ± 10.1	33.7 ± 7.8

[a] Type II-R: Galβ1,4GlcNAc-$O(CH_2)_8CO_2CH_3$.

[b] Type I-R: Galβ1,3GlcNAc-$O(CH_2)_8CO_2CH_3$.

[c] NA: not applicable.

Fig. 2-4. Schematic representations of chimeric FucTs (A) and their α1,3 and α1,4 activities (B). **(A)** Top: The bar represents the FucT protein. The two catalytic domains are shown in vertical shaded blocks. The three highly divergent regions are boxed. Bottom: Schematic bars represent the structure of chimeric FucTs. The length (number of total amino acids) of each FucT construct is given at the right side of each bar. **(B)** Enzyme activities (mU mg^{-1}) for α1,3-FucT activity (gray bars, bottom Y-axis) and α1,4-FucT activity (dark blue bars, top Y-axis) of each FucT construct are standardized by FucT expression level. Each value represents the average of three or four determinations with standard deviations indicated. The ratio of α1,4/α1,3 activity (%) for each FucT construct is shown on the right.


A
B
FucT
I
II
repeats
WT and Chimeric FucTs
Length of FucT
11639FucT
UA948FucT
$11639^{1-110}UA948^{111-462}$
$11639^{1-158}UA948^{157-462}$
$11639^{1-296}UA948^{295-462}$
$11639^{1-359}UA948^{361-462}$
$UA948^{1-110}11639^{111-478}$
$UA948^{1-156}11639^{159-478}$
$UA948^{1-294}11639^{297-478}$
$UA948^{1-360}11639^{360-478}$
$11639^{347CNDAHYSALH}$
$UA948^{345DNPFIFC}$
$11639^{1-110}UA^{111-294}11639^{297-478}$
$11639^{1-158}UA^{157-360}11639^{360-478}$
478
462
462
464
464
461
478
476
476
479
479
459
476
481
α1,4 Activity (mU mg^{-1})
0
5
10
15
20
α1,3
α1,4
α1,3 Activity (mU mg^{-1})
0
10
20
30
40
50
60
α1,4/α1,3 (%)
0
31
17
15
5
0.1
0.2
0
0
24
7
0.4
0.3
10

according to the FucT protein expression level of WT and chimeric FucTs are given in Fig. 2-4B. In addition, the α1,4/α1,3 activity ratio (%) was calculated (Fig. 2-4B).

The α1,3 activity of 11639FucT was 14.2 mU mg^{-1}, which is lower than that of UA948FucT (23.8 mU mg^{-1}). The α1,4 activity of UA948FucT was one third of its α1,3 activity. Constructs 11639$^{1-110}$UA948$^{111-462}$, 11639$^{1-158}$UA948$^{157-462}$ and 11639$^{1-296}$UA948$^{295-462}$ containing the 11639FucT N-terminus and the UA948FucT C-terminus had both α1,3 and α1,4 FucT activities. In contrast, their counterparts, constructs UA948$^{1-110}$11639$^{111-478}$, UA948$^{1-156}$11639$^{159-478}$ and UA948$^{1-294}$11639$^{297-478}$, containing the UA948FucT N-terminus and the 11639FucT C-terminus, displayed α1,3 activity with little or no α1,4 activity. Therefore, it is the C-terminus that controls Type I or Type II acceptor specificity in *H. pylori* FucTs. This is different from human FucTs, where the N-terminal hypervariable stem domain determines acceptor specificity. UA948$^{1-156}$11639$^{159-478}$ and UA948$^{1-294}$11639$^{297-478}$ exhibited very low α1,3 FucT activity, although they were stably expressed (Fig. 2-3). Apparently the two segments of the chimera are not fully compatible in these cases, which results in the low enzyme activity.

Mutant 11639$^{1-359}$UA948$^{361-462}$ displayed α1,3 activity at WT level without gaining α1,4 activity, indicating that the C-terminal heptad repeats of UA948FucT cannot confer Type I acceptor specificity. This is further supported by the substitution of heptad repeats of UA948FucT by those of 11639FucT (construct UA948$^{1-360}$11639$^{360-478}$), which failed to abolish the α1,4 activity. However, although chimera UA948$^{1-360}$11639$^{360-478}$ exhibited a near WT UA948FucT α1,4/α1,3 activity ratio (24% versus 31%), both its α1,3 and α1,4 activities were low. Apparently, the heptad repeats of 11639FucT have a negative impact on catalysis by the UA948 catalytic domain, affecting α1,3 and α1,4 activities to a similar extend. In contrast, the 11639FucT catalytic domain can tolerate the exchange of heptad repeats by those of UA948 FucT.

Mosaic construct $11639^{1\text{-}110}$UA$948^{111\text{-}294}11639^{297\text{-}478}$ had normal α1,3 activity but no α1,4 activity, demonstrating that the internal region (110-294) of UA948FucT was unable to confer α1,4 activity either. In contrast, construct $11639^{1\text{-}158}$UA$948^{157\text{-}360}11639^{360\text{-}478}$, exhibited both α1,3 and α1,4 activity, though both at low level. Comparing all ten chimeras it becomes obvious that those containing residues 295-361 of UA948FucT have α1,4 activity, whereas all without it lack α1,4 activity. Therefore, it is likely that residues in this segment confer α1,4 activity. The segment (295-361) includes the second divergent region. Remarkably, chimera $11639^{347\text{CNDAHYSALH}}$ gained 11% of WT UA948FucT α1,4 activity. In contrast, its counterpart UA$948^{345\text{DNPFIFC}}$ lost almost all of its α1,4 activity without losing its α1,3 activity. This suggests that the second divergent region determines the absence or presence of α1,4 activity. The data indicate that residues ^{345}CNDAHYSALH^{354} of UA948FucT are essential for conferring Type I substrate specificity.

It should be noted that $11639^{347\text{CNDAHYNALH}}$ gained relatively low α1,4 activity and its α1,4/α1,3 ratio is also lower than that of WT UA948FucT, suggesting that some other residues may further contribute to acceptor substrate specificity. UA$948^{1\text{-}360}11639^{360\text{-}478}$, but not $11639^{1\text{-}296}$UA$948^{295\text{-}462}$, has a near WT α1,4/α1,3 ratio, suggesting that additional acceptor specificity determinants are located at the N-terminus, prior to residue 296. Chimera $11639^{1\text{-}110}$UA$948^{111\text{-}462}$ displays a two-fold drop in α1,4/α1,3 ratio which does not deteriorate further in the $11639^{1\text{-}158}$UA$948^{157\text{-}462}$ construct. This suggests that the additional acceptor specificity determinants reside in the N-terminal 110 residues.

However, at this moment a role for residues 158 to 296 cannot be ruled out since chimera $11639^{1\text{-}158}UA948^{157\text{-}462}$ has a noticeable higher α1,4/α1,3 ratio than chimera $11639^{1\text{-}296}UA948^{295\text{-}462}$. The data indicate that the second divergent region is crucial for α1,4 activity but more detailed studies will be needed to delineate the additional acceptor determinants more precisely.

2.3.5 Kinetics Parameters of Mutants

The kinetic parameters of two WT FucTs and constructs $11639^{1\text{-}359}UA948^{361\text{-}462}$, $UA948^{1\text{-}360}11639^{360\text{-}478}$, $11639^{347CNDAHYNALH}$ and $UA948^{345DNPFIFC}$ are shown in Table 2-3. The α1,4 activity of $11639^{1\text{-}359}UA948^{361\text{-}462}$ was too low to obtain reliable kinetic parameters.

The $V_{\max}$ of 11639FucT using Type II acceptor was about one third of that of UA948FucT, but the $V_{\max} / K_m$ of 11639FucT was higher than that of UA948FucT due to its lower K_m. Therefore, 11639FucT has a higher fucose transfer efficiency in α1,3-linkage than UA948FucT. Type II is a better acceptor than Type I for UA948FucT because of its higher fucose incorporation ($V_{\max}$) and lower K_m. Similar acceptor K_m values were obtained for $11639^{1\text{-}359}UA948^{361\text{-}462}$ and $UA948^{1\text{-}360}11639^{360\text{-}478}$ compared to their corresponding WT enzymes, which confirmed that the C-terminal heptad repeats are not responsible for acceptor recognition.

In comparison to WT 11639FucT, $11639^{347CNDAHYNALH}$ possessed a higher K_m for Type II acceptor (2.2 mM), which was close to the K_m value of UA948FucT. Similarly, the K_m value of $UA948^{345DNPFIFC}$ for Type II was reduced from 2.2 mM to 0.17 mM which is close to that of 11639FucT (0.37 mM). This strongly implies that the second

Table 2-3. Acceptor and donor kinetic parameters of wild type and chimeric FucTs. Acceptor kinetics were determined in cell crude extracts using 0.03-2 mM Type II-R, or 0.4-25 mM Type I-R with GDP-fucose at 200 μM and GDP-^{3}[H]Fuc at 0.2 μM. Donor kinetics were determined using 3-200 μM GDP-fucose with Type II-R at 2 mM or Type I-R at 15 mM and GDP-^{3}[H]Fuc at 0.2 μM.

	Type II-R[b]			Type I-R[c]			K_m (μM) for GDP-fucose	
WT/Mutants[a]	K_m (mM)	V_{max} [e]	V_{max} / K_m [d]	K_m (mM)	V_{max} [e]	V_{max} /Km[d]	Type II-R[b]	Type I-R[d]
11639	0.37 ± 0.03	11.8 ± 0.3	31.9	NA[f]	NA[f]	NA[f]	44.7 ± 5.5	NA[f]
UA948	1.5 ± 0.2	29.0 ± 1.8	19.3	13.4 ± 1.4	11.7 ± 0.6	0.87	56.0 ± 10.1	33.7 ± 7.8
$11639^{1-359}UA948^{361-462}$	0.7 ± 0.1	41.6 ± 0.01	48.5	ND[g]	ND[g]	ND[g]	153 ± 8	ND[g]
$UA948^{1-360}11639^{360-478}$	1.3 ± 0.2	4.1 ± 0.3	3.1	9.7 ± 1.7	1.1 ± 0.08	0.11	236 ± 47	188 ± 20
$11639^{347CNDAHYSALH}$	2.2 ± 0.6	8.8 ± 1.5	4.0	20.8 ± 5.9	0.7 ± 0.1	0.03	244 ± 34	54.6 ± 6.6
$UA948^{345DNPFIFC}$	0.17 ± 0.01	28.9 ± 0.6	170.0	22.7 ± 7.4	0.7 ± 0.1	0.03	48.1 ± 7.0	213 ± 52

[a] All FucTs possessed a His$_6$-tag at the C-terminus.

[b] Type II-R: Galβ1,4GlcNAc-O$(CH_2)_8CO_2CH_3$.

[c] Type I-R: Galβ1,3GlcNAc-O$(CH_2)_8CO_2CH_3$.

[d] V_{max} / K_m : mU mg^{-1} mM^{-1}.

[e] V_{max} (mU mg^{-1}) was standardized based on FucT expression detected by Western Blot.

[f] NA: not applicable.

[g] ND: not determined, the activity was too low to determine the kinetic parameters with confidence.

divergent region plays an essential role in Type II acceptor recognition. In addition, chimera11639^{347CNDAHYNALH} gained α1,4 activity with the K_m value for Type I substrate near the WT UA948FucT level. This verifies the vital role of those ten amino acids in conferring α1,4 activity. On the other hand, as expected, the catalysis of Type I substrate was severely diminished in the UA948^{345DNPFIFC} chimera, despite the fact that acceptor K_m of UA948^{345DNPFIFC} for acceptor Type I was similar to the level of WT UA948FucT, the V_{max} of this chimera was dramatically decreased. This suggests that those ten amino acids present in UA948FucT play an important role in efficient fucose transfer in α1,4-linkage.

2.4 Discussion

Mammalian α1,3/4 FucTs, especially human FucTs, have been extensively characterized, with regard to the key polar-group of the acceptor (de Vries *et al.*, 1995; De Vries *et al.*, 1997; Du and Hindsgaul, 1996; Gosselin and Palcic, 1996), the domain or amino acid residues responsible for donor binding (Holmes *et al.*, 1995; Holmes *et al.*, 2000; Jost *et al.*, 2005; Kimura *et al.*, 1997; Sherwood *et al.*, 1998; Sherwood *et al.*, 2002; Shinoda *et al.*, 1998) or acceptor recognition (De Vries *et al.*, 1997; de Vries *et al.*, 2001b; Dupuy *et al.*, 1999; Dupuy *et al.*, 2004; Jost *et al.*, 2005; Legault *et al.*, 1995; Nguyen *et al.*, 1998; Sherwood *et al.*, 2002; Vo *et al.*, 1998), and kinetic mechanisms (Murray *et al.*, 1996; Murray *et al.*, 1997; Qiao *et al.*, 1996). The study of bacterial α1,3/4 FucTs, in contrast, is not as far advanced. To date, bacterial α1,3/4 FucTs have only been characterized in *H. pylori* (Ge *et al.*, 1997; Martin *et al.*, 1997; Rasko *et al.*, 2000a; Rasko *et al.*, 2000b) and *Vibrio cholerae* (Stroeher *et al.*, 1997), despite identification of putative FucT homologs in *Rickettsia conorii* (Ogata *et al.*, 2000), *Salmonella enterica serovar Typhi* (McClelland *et al.*, 2001; Parkhill *et al.*, 2001a), *Yersinia pestis* (Parkhill *et al.*, 2001b) and *Mesorhizobium loti* (Sullivan *et al.*, 2002).

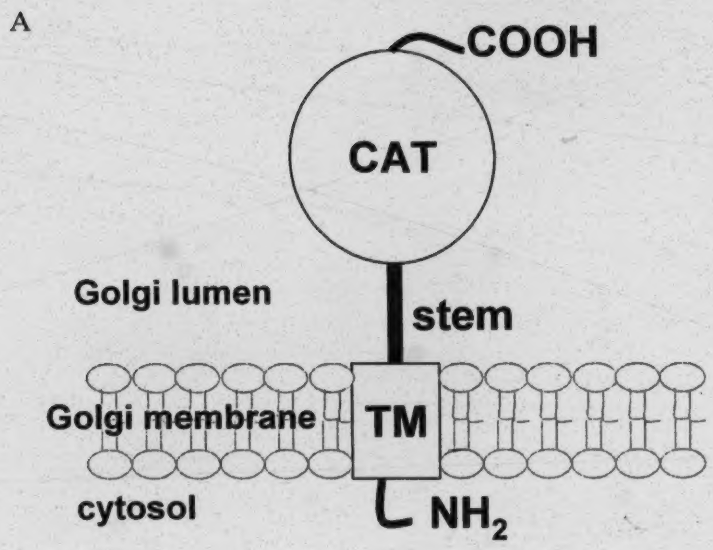
The aim of this study was to identify the determinants of *H. pylori* α1,3/4 FucTs that confer recognition of Type I and Type II substrates. The data demonstrate that the C-terminal region of *H. pylori* FucTs controls Type I and Type II substrate recognition. Particularly, residues ^{345}CNDAHYSALH^{354} of UA948FucT are essential for α1,4 activity. It is worth mentioning that the donor binding affinity was modified in constructs 11639$^{1\text{-}359}$UA948$^{361\text{-}462}$, UA948$^{1\text{-}360}$11639$^{360\text{-}478}$ and 11639$^{347\text{CNDAHYSALH}}$ with Type II-R acceptor and UA948$^{345\text{DNPFIFC}}$ with Type I-R acceptor. It is most likely that the binding of *H. pylori* FucT enzyme with acceptor and donor is a coordinated process. The recently published crystal structures of catalytic domains from other families of GTs demonstrate that for both inverting enzymes, bovine β1,4-galactosyltransferase (Ramakrishnan and Qasba, 2002) and bovine N-acetylglucosaminyltransferase I (Unligil *et al.*, 2000), as well as retaining enzymes, bovine α1,3-galactosyltransferase (Boix *et al.*, 2002), α1,4-galactosyltransferase (Persson *et al.*, 2001), human galactosyltransferase and human N-acetylgalactosaminyltransferase (Patenaude *et al.*, 2002), the donor binding caused a conformational change that was essential to create the acceptor binding site. The data suggest that these enzymes followed a sequential ordered bi-bi catalytic mechanism. The same mechanism was reported for *E. coli* MurG (Chen *et al.*, 2002) and human FucT V (Qiao *et al.*, 1996) based on product inhibition studies. The donor K_m modification of chimeras observed in the current study implies that *H. pylori* FucTs might follow the same mechanism. However, it is also possible that the domain swapping experiments cause significant folding changes, which could affect donor and acceptor binding. More detailed kinetic studies and/or crystallography structure analysis would clarify this issue.

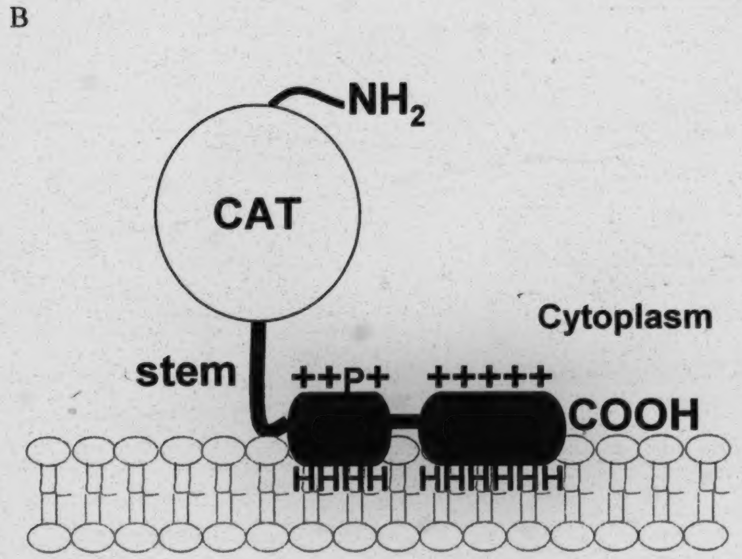
To date, FucT enzymes that display α1,4 activity have also been found in the chimpanzee (Costache *et al.*, 1997), Rhesus macaque (Old World Monkey) (Dupuy *et al.*, 2002) and plants (Bakker *et al.*, 2001; Leonard *et al.*, 2002). According to sequence alignment, all these FucTs contain a tryptophan residue within the N-terminal hypervariable stem region, which was found to be essential for conferring α1,4 activity in mammalian FucTs (Dupuy *et al.*, 1999). This tryptophan residue is conserved in human FucT III and FucT V, which have both α1,3 and α1,4 activities; but is not present in other human FucTs possess only α1,3 activity. In *H. pylori*[2], UA1111FucTa and UA1111FucTb also display α1,4 activity, though their activities are very much lower than that of UA948FucT (Rasko *et al.*, 2000a). Neither of these two FucTs contains those ten crucial amino acids ^{345}CNDAHYSALH^{354} that are present in UA948FucT. Instead, UA1111FucTa has ^{346}DNPFIFY^{352}, which resembles the majority of *H. pylori* FucTs (Fig. 2-2); whereas UA1111FucTb has ^{347}KSSTSFMWE^{355} (Rasko *et al.*, 2000a), which are similar to 26695FucTa (Fig. 2-2). Apparently, acceptor specificity determinants in *H. pylori* FucTs are not as conserved as in eukaryotic FucTs. The residues conferring α1,4 activity in UA1111FucTa and UA1111FutTb remain to be determined.

The results in the current study demonstrate that the C-terminal region of *H. pylori* FucTs controls Type I and Type II substrate recognition. In particular, residues ^{345}CNDAHYSALH^{354} of UA948FucT are essential for α1,4 activity, whereas the 110 N-terminal residues may further contribute to α1,4 activity. In complete contrast, acceptor specificity in human FucTs is determined by the residues at the N-terminal hypervariable stem domain (Dupuy *et al.*, 1999; Legault *et al.*, 1995; Nguyen *et al.*, 1998; Xu *et al.*, 1996), with possibly a further contribution by C-terminal residues (Vo *et al.*, 1998). However, in spite of the different location of the acceptor specificity determinants in mammalian and *H. pylori* FucTs, sequence analysis suggests that they may be more similar on a structural level in terms of domain organization.

Analysis of the C-terminal sequence of *H. pylori* FucTs showed two remarkable features: the heptad repeats and two conserved regions rich in positive and hydrophobic residues (in brackets in Fig. 2-2). The C-terminal heptad repeats are predicted to fold into an α-helix (PepTool) and it has been suggested that the heptad repeats form a leucine zipper motif that may serve as a dimerization domain (Ge *et al.*, 1997; Martin *et al.*, 1997). Deletion of the C-terminal 99 amino acids of 11639FucT has been reported to abolish enzyme activity (Ge *et al.*, 1997), suggesting that the heptad repeats do play a role in the enzyme function.

The patterns of positive and hydrophobic residues in the C-terminal tail region, KIYRK(A/I)YQK and LLR(A/V/T)(I/V)RR(W/L)V(K/R)K (in brackets in Fig. 2-2) perfectly match the requirements to form extremely amphipathic helices with positive and hydrophobic residues occupying the opposite faces of the helix (Fig 2-5). Such helices can act as membrane anchors, with their hydrophobic face embedded in the membrane and the positive charges interacting with phospholipids headgroups (Chen *et al.*, 1999a; Wakarchuk *et al.*, 1998). Notably, the C-terminal 50 amino acids of *Neisseria meningitidis* α1,4 galactosyltransferase are also rich in positively charged and hydrophobic residues and have been found to be responsible for membrane association


A
COOH
CAT
Golgi lumen
stem
Golgi membrane
TM
cytosol
NH_2


B
NH_2
CAT
Cytoplasm
stem
++P+
+++++
COOH
HHHH
HHHHHH

(Persson *et al.*, 2001). Finally, the existence of a membrane anchor also fits the experimental data that *H. pylori* FucTs are membrane associated (Ge *et al.*, 1997).

The sequence properties described above suggest that two amphipathic helices at the C-terminus of *H. pylori* FucTs could correspond to the N-terminal membrane anchor of mammalian FucTs. Similarly, the heptad repeat region of *H. pylori* FucTs could act as a spacer linking the catalytic domain to the membrane anchor, corresponding in function to the stem region of mammalian FucTs. This suggests that the domains of mammalian FucTs and *H. pylori* FucTs are organized in a similar manner: a membrane anchor is linked by a hypervariable stem region to the catalytic domain (Fig. 2-5). As a result, the substrate recognition sites for both FucT families are located at their hypervariable stem region. Similarly, the stem region of β-galactoside-α2,6-sialyltransferase, another family of GTs, was also found to govern the preference for glycoprotein acceptors (Legaigneur *et al.*, 2001).

In conclusion the substrate specificity determinants of *H. pylori* FucTs was characterized. Although there is only a low level of sequence identity between mammalian FucTs and *H. pylori* FucTs, a putative similarity in their structural organization is discovered. As a result, acceptor specificity may in both cases be determined by divergent sequences in the stem region. However, the molecular basis for acceptor specificity remains to be determined by further structure and function studies, ideally in combination with a crystal structure.

2.5 Acknowledgments

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2.6 Footnotes

[1] 11637 variant 3a was recently found to be Le^a positive as detected by ELISA (Appelmelk *et al.*, 2000), leading to the authors' suggestion that FucT in this strain may have α1,4 activity, which remains to be confirmed by the enzyme assay.

CHAPTER 3

A Single Aromatic Amino Acid at the C-terminus of *Helicobacter pylori* α1,3/4 Fucosyltransferase Determines Substrate Specificity

A version of this work has been published previously[1]:

Ma, B. Lau, L. H. Palcic, M. M. Hazes, B and Taylor, D. E. A single aromatic amino acid at the C-terminus of *Helicobacter pylori* α1,3/4 fucosyltransferase determines substrate specificity. *J. Biol. Chem.* 2005 **280**:36848-36856.

[1]: Capillary electrophoresis section was performed by Leon H. Lau.

3.1 Introduction

H. pylori is associated with gastritis and peptic ulcer formation and is a risk factor for the development of gastric cancer and MALT lymphoma. One of the virulence factors of *H. pylori* is the LPS, which contains lipid A, core oligosaccharide and O-antigens. The O-antigens of *H. pylori* LPS contain fucosylated oligosaccharides, predominantly the Type II blood group antigens, Le^x (Galβ1,4(Fucα1,3)GlcNAc) and Le^y (Fucα1,2Galβ1,4(Fucα1,3)GlcNAc) (Wirth *et al.*, 1996), but a small number of *H. pylori* strains also express the Type I blood group antigens, Le^a (Galβ1,3(Fucα1,4)GlcNAc) and Le^b (Fucα1,2Galβ1,3(Fucα1,4)GlcNAc) (Monteiro *et al.*, 1998a).

The role of Lewis antigens in *H. pylori* pathogenesis is still ambiguous. It has been suggested that Lewis antigens play a role in *H. pylori* adhesion to (Appelmelk *et al.*, 2000; Edwards *et al.*, 2000) or internalization by (Lozniewski *et al.*, 2003) the gastric epithelial cells. Nevertheless, conflicting evidence argues that Le^x and Le^y are not required for colonization of human gastric epithelium (Mahdavi *et al.*, 2003) or mouse stomach (Suresh *et al.*, 2000; Takata *et al.*, 2002). Lewis antigens may also play an important role in the persistence of *H. pylori* infection by molecular mimicry, helping the bacteria to evade the host immune response (Monteiro *et al.*, 1998a; Moran *et al.*, 1996; Moran *et al.*, 2002). Environmental changes such as pH influence the expression of *H. pylori* O-antigens, particularly Le^x and Le^y. This may aid in adaptation of the bacterium to its niche in the stomach (Moran *et al.*, 2002).

FucTs are enzymes responsible for the last steps in the synthesis of Lewis antigens in *H. pylori* (Appelmelk *et al.*, 1999; Wang *et al.*, 2000). α1,2 and α1,3 or α1,3/4 FucTs have been identified and characterized in *H. pylori* (Ge *et al.*, 1997; Martin *et al.*, 1997;

Rabbani *et al.*, 2005; Rasko *et al.*, 2000a; Rasko *et al.*, 2000b; Wang *et al.*, 1999a; Wang *et al.*, 1999b). These FucTs catalyze the transfer of the L-fucose moiety from guanosine diphosphate β-L-fucose (GDP-Fuc) to the OH-2 of the galactose moiety, and to the OH-3 position alone, or to both OH-3 and OH-4 positions of the GlcNAc moiety in glycoconjugate acceptors, respectively. The *H. pylori* genome contains two homologous α1,3/4 FucT genes, *futA* and *futB* (Alm *et al.*, 1999; Tomb *et al.*, 1997), but they do not always encode functional proteins. For instance, only the *futA* gene encodes an active FucT in *H. pylori* strains NCTC11639 and UA948 (Ge *et al.*, 1997; Martin *et al.*, 1997).

Bacterial FucTs are functionally equivalent to the mammalian FucTs, which have been well characterized. Mammalian FucTs are Type II membrane proteins with a short N-terminal cytoplasmic tail, transmembrane domain, stem region and C-terminal catalytic domain. *H. pylori* FucTs share weak homology with their mammalian counterparts in two small segments within the catalytic domain, called α1,3 FucT motifs (Martin *et al.*, 1997; Oriol *et al.*, 1999). *H. pylori* α1,3/4 FucTs lack the N-terminal domain that attaches the mammalian enzyme to the membrane. Instead, they contain 2 to 10 heptad repeats that connect the N-terminal catalytic domain with two amphipathic helices at the C-terminus (Rasko *et al.*, 2000b). These C-terminal heptad repeats and amphipathic helices are believed to be functionally equivalent to the N-terminal stem and transmembrane regions of mammalian FucTs, respectively (Ma *et al.*, 2003).

Mammalian α1,3/4 FucTs exhibit distinct Type I and Type II acceptor preferences. Human FucT III, V, VI and bovine FucT share considerable protein sequence identity (>70%) (Oulmouden *et al.*, 1997; Weston *et al.*, 1992), but they display different acceptor specificity patterns. FucT VI and bovine FucT possess exclusively α1,3 activity, whereas

FucT III and V have both α1,3 and α1,4 activity. FucT III favors Type I acceptors, therefore it is predominantly an α1,4 FucT; whereas FucT V prefers Type II acceptors hence it is primarily an α1,3 FucT (Costache *et al.*, 1997). Domain swapping experiments performed between FucT III and VI (Legault *et al.*, 1995) or between FucT III and V (Nguyen *et al.*, 1998) demonstrated that the N-terminal hypervariable stem region in human FucTs determines acceptor specificity. In particular, Trp^{111} in human FucT III was discovered to be responsible for Type I acceptor recognition (Dupuy *et al.*, 1999; Dupuy *et al.*, 2004). The Trp residue is also present in FucT V. In contrast, the α1,3 FucTs including human FucT IV, VI, VII and IX and bovine FucT have an Arg at the corresponding position.

Similar to mammalian FucTs, *H. pylori* α1,3/4 FucTs also exhibit Type I and Type II acceptor specificity. Radiochemical assays showed that 11639FucT is an α1,3 FucT (Ge *et al.*, 1997), whereas UA948FucT displays both α1,3 and α1,4 activity with a preference for Type II acceptor (Ma *et al.*, 2003; Rasko *et al.*, 2000b). In an effort to identify the region that determines acceptor specificity, 12 chimeric FucTs were constructed by domain swapping between 11639FucT and UA948FucT in the previous study. In contrast to mammalian FucTs, the data demonstrated that exchange of a small hypervariable region near the C-terminus, specifically $^{347}DNPFIFC^{353}$ in 11639FucT and $^{345}CNDAHYSALH^{354}$ in UA948FucT, was sufficient to either confer or abolish α1,4 activity (Ma *et al.*, 2003). The goal of the current investigation is to identify specific amino acids within $^{345}CNDAHYSALH^{354}$ of UA948FucT that control Type I acceptor recognition.

3.2 Experimental Procedures

3.2.1 Materials

Primers for site-directed mutagenesis were synthesized by Invitrogen Canada Co. (Burlington, Ontario). *Pfu* turbo DNA polymerase was purchased from Stratagene (La Jolla, CA). pGEM-T vector was obtained from Promega Co. (Madison, WI). Type II-R (Galβ1,4GlcNAc-O-$(CH_2)_8CO_2CH_3$), Type I-R (Galβ1,3GlcNAc-O-$(CH_2)_8CO_2CH_3$) and Type I-tetramethylrhodamine (Galβ1,3GlcNAc-O-$(CH_2)_8$CO-$NHCH_2CH_2NH$-TMR) were kindly provided by Dr. Ole Hindsgaul. C_{18} Sep-Pak cartridge was obtained from Waters (Milford, MA). α1,3/4-Fucosidase was purchased from CalBiochem-NovaBiochem (La Jolla, CA). Anti-penta-histidine monoclonal antibody, plasmid mini-preparation and midi-preparation kits were purchased from QIAGEN Co. (Mississauga, ON). GDP-fucose and horse-radish peroxidase conjugated goat anti-mouse IgG were from Sigma Chemical Co. (St Louis, MO). GDP-^{3}[H]Fuc (0.1 mCi ml^{-1}, 17.3 Ci $mmol^{-1}$) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). The BCA protein assay kit was purchased from Pierce Biotech Inc. (Rockford, IL). Nitrocellulose membrane was obtained from Micron Separation Inc. (Westboro, MA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Biosciences Corp. (Buckinghamshire, England). BioMax MR film was obtained from Eastman Kodak Co. (Rochester, NY).

3.2.2 Mutant Construction

WT *futA* gene of 11639FucT or UA948FucT was cloned into pGEM-T vector with a His_6-tag fused at the C-terminal end (Ma *et al.*, 2003). Using plasmid DNA harboring the *futA* gene as DNA template, alanine substitutions were created by PCR following the QuickChange^{TM} site-directed mutagenesis protocol from Stratagene (La Jolla, CA). DNA manipulations were performed in *E. coli* XL1-blue (Stratagene, La Jolla, CA). The sequences of the primers used for constructing the mutants are shown in Table 3-1. Each mutant was sequenced to confirm the desired single amino acid mutation. No unexpected mutations occurred during their construction.

3.2.3 Induction and Expression of *H. pylori* FucT Genes

WT and mutant FucT proteins were expressed in *E. coli* HMS174DE3 cells as described previously (Ma *et al.*, 2003).

3.2.4 Standard Fucosyltransferase Assay

FucT enzyme activities were assayed in reactions containing donor GDP-fucose at 200 μM, GDP-^{3}[H]Fuc (60,000 dpm), and Type II-R or Type I-R acceptors at concentrations of 1.8 mM or 7.5 mM, respectively. The reverse-phase C_{18} Sep-Pak cartridge was used to isolate the products (containing the hydrophobic aglycone and the acquired radiolabeled fucose moiety) from the un-reacted GDP-^{3}[H]Fuc as described previously (Palcic, 1988). One milliunit (mU) represents the amount of enzyme that converts 1 nmol of acceptor substrate to product per min. The specific activity (mU mg^{-1}) was obtained by dividing the enzyme activity (mU) by the amount of total protein that was determined by BCA protein assay kit. Specific enzyme activity below 0.01 mU mg^{-1} was considered undetectable.

3.2.5 Immuno-blot Analysis of WT and Mutant FucT Protein Expression

Cell extracts containing equal amounts of total protein (7.35 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred to nitrocellulose membrane (pore size, 0.22 μm). Nitrocellulose blots were probed with mouse anti-penta-histidine monoclonal primary antibody (1:1000)

Table 3-1. Sequences of primers used for site-directed mutagenesis. Primer names begin with the *H. pylori* strain to be mutated, UA948 or 11639, followed by the wild type residue, location, mutated residue ending with an "F" or "R" to designate sense and anti-sense, respectively. Mutated base pairs with the primer sequences are shown in lower case.

Primer's name	Primer sequence
UA948C345AF	5' GAA AAC GAT ACG ATT TAT CAT gcC AAT GAT GCC CAT TAT TCT GC 3'
UA948C345AR	5' GC AGA ATA ATG Ggc ATG ATT GGC ATG ATA AAT CGT ATC GTT TTC 3'
UA948N346AF	5' C GAT ACG ATT TAT CAT TGC gcT GAT GCC CAT TAT TCT GCT CTT C 3'
UA948N346AR	5' G AAG AGC AGA ATA ATG GGC ATC Agc GCA ATG ATA AAT CGT ATC G 3'
UA948D347AF	5' GAT ACG ATT TAT CAT TGC AAT GcT GCC CAT TAT TCT GCT CTT 3'
UA948D347AR	5' AAG AGC AGA ATA ATG GGC AgC ATT GCA ATG ATA AAT CGT ATC 3'
UA948H349AF	5' CG ATT TAT CAT TGC AAT GAT GCC gcT TAT TCT GCT CTT CAT CG 3'
UA948H349AR	5' CG ATG AAG AGC AGA ATA Agc GGC ATC ATT GCA ATG ATA AAT CG 3'
UA948Y350AF	5' CAT TGC AAT GAT GCC CAT gcT TCT GCT CTT CAT CGT G 3'
UA948Y350AR	5' C ACG ATG AAG AGC AGA Agc ATG GGC ATC ATT GCA ATG 3'
UA948S351AF	5' GC AAT GAT GCC CAT TAT gCT GCT CTT CAT CGT GAT TTG 3'
UA948S351AR	5' CAA ATC ACG ATG AAG AGC AGc ATA ATG GGC ATC ATT GC 3'
UA948L353AF	5' C AAT GAT GCC CAT TAT TCT GCT gcT CAT CGT GAT TTG AAT GAG CC 3'
UA948L353AR	5' GG CTC ATT CAA ATC ACG ATG Agc AGC AGA ATA ATG GGC ATC ATT G 3'
UA948H354AF	5' GCC CAT TAT TCT GCT CTT gcT CGT GAT TTG AAT GAG CCG 3'
UA948H354AR	5' CGG CTC ATT CAA ATC ACG Agc AAG AGC AGA ATA ATG GGC 3'
UA948Y350FF	5' CAT TGC AAT GAT GCC CAT TtT TCT GCT CTT CAT CGT G 3'
UA948Y350FR	5' C ACG ATG AAG AGC AGA AaA ATG GGC ATC ATT GCA ATG 3'
UA948Y350GF	5' CAT TGC AAT GAT GCC CAT ggT TCT GCT CTT CAT CGT G 3'
UA948Y350GR	5' C ACG ATG AAG AGC AGA Acc ATG GGC ATC ATT GCA ATG 3'
UA948Y350WF	5' CAT TGC AAT GAT GCC CAT Tgg TCT GCT CTT CAT CGT G 3'
UA948Y350WR	5' C ACG ATG AAG AGC AGA ccA ATG GGC ATC ATT GCA ATG 3'
11369F350YF	5' CG ATT TAT CAC GAT AAC CCT TaC ATT TTC TGT CGT GAT TTG 3'
11639F350YR	5' CAA ATC ACG ACA GAA AAT GtA AGG GTT ATC GTG ATA AAT CG 3'
11639F352YF	5' CAC GAT AAC CCT TTC ATT TaC TGT CGT GAT TTG AAT GAG CC 3'
11639F352YR	5' GG CTC ATT CAA ATC ACG ACA GtA AAT GAA AGG GTT ATC GTG 3'
11639F350AF	5' CG ATT TAT CAC GAT AAC CCT gcC ATT TTC TGT CGT GAT TTG AAT G 3'
11639F350AR	5' C ATT CAA ATC ACG ACA GAA AAT Ggc AGG GTT ATC GTG ATA AAT CG 3'
11639F352AF	5' CAC GAT AAC CCT TTC ATT gcC TGT CGT GAT TTG AAT GAG CC 3'
11639F352AR	5' GG CTC ATT CAA ATC ACG ACA Ggc AAT GAA AGG GTT ATC GTG 3'

and goat anti-mouse secondary antibody conjugated with horse-radish peroxidase (1:2000). Blots were developed using an enhanced chemiluminescence (ECL) kit and the images were visualized on BioMax MR films. A standard curve plotting the band density versus the amount of protein loaded for immunoblot analysis was carried out in tge previous study and 7.35 μg of total protein was shown in the middle of the linear range of the standard curve (Ma *et al.*, 2003). The density of each FucT band was quantified using Alpha Ease FC software (Alpha Innotech Corporation, San Leandro, CA), and the expression level of WT UA948FucT was set to 1. Subsequently, the expression level of each FucT mutant was determined relative to that of UA948FucT and used to normalize the enzyme specific activity, by dividing the enzyme specific activity (mU mg^{-1}) by the relative FucT expression level.

3.2.6 Capillary Electrophoresis Assay

To detect very low levels of α1,4 activity in FucT enzymes, capillary electrophoresis (CE) was used in the current study. The incubation mixtures contained 6.3 μl of the cell crude extract, 10 μM Type I-TMR, 200 μM GDP-Fucose in a total volume of 10 μl including 20 mM HEPES (pH 7.0), 100 mM NaCl, 35 mM $MgCl_2$, 1 mM ATP, 5 mg ml^{-1} BSA and 20 mM $MnCl_2$. The reaction mixtures were incubated at 37°C for 30 min, 1 h, 2 h or 5 h. Samples were removed and diluted (1:25) with CE running buffer (10 mM phosphate, 10 mM borate, 10 mM SDS and 10 mM phenylboronic acid, pH 9.0) to quench the reaction. Samples were subsequently electrokinetically injected onto the electrophoresis capillary (40 cm long and 9 μm internal diameter) (Polymicro Technologies, Phoenix, AZ) at 4 kV for 4 s. Products were analyzed by capillary electrophoresis at 450 V cm^{-1} as described previously (Chan *et al.*, 1995; Ge *et al.*, 1997).

The area of the product and substrate peaks were integrated by Igor Pro software (Lake Oswego, OR), and compared to each other to give a quantitative analysis of each reaction. The percentage of Type I-TMR conversion to Lewis A-TMR in each reaction was standardized with the total protein concentration as well as the FucT expression level that was detected by Western blot. The total protein concentration and the FucT expression from WT UA948FucT was set as 1, the relative levels of total protein concentration and FucT expression of each mutant were determined relative to those of WT UA948FucT.

The α-1,3/4-fucosidase treatment was performed by removing 4 μl of the 5 h reaction mixtures and heating up to 90°C for 10 min to de-activate residual FucT activity. The mixtures were then incubated with 20 microunits of α-1,3/4-fucosidase in a total volume of 40 μl of 50 mM sodium phosphate buffer (pH 5.0) at 37°C for 6 h. Products were analyzed by capillary electrophoresis. The percentage of Lewis A-TMR present in each reaction was quantified and standardized with total protein concentration and FucT expression level as described above.

3.2.7 Determination of Kinetic Parameters

Acceptor kinetics were performed using 0.03-2 mM Type II-R, or 0.4-25 mM Type I-R, with GDP-fucose at 200 μM including GDP-^{3}[H]Fuc at 0.2 μM. 2 mM of Type II-R is the highest concentration that can be reached in current study due to its low solubility. Due to the limited supply of the acceptors, donor kinetics were determined using 3-200 μM GDP-fucose with Type II-R at 2 mM or Type I-R at 15 mM including GDP-^{3}[H]Fuc at 0.8 μM. Kinetic parameters were obtained by fitting the initial rate data to the

Michaelis-Menten equation using non-linear regression analysis with Prism 4.0 software (GraphPad, San Diego, CA).

3.3 Results

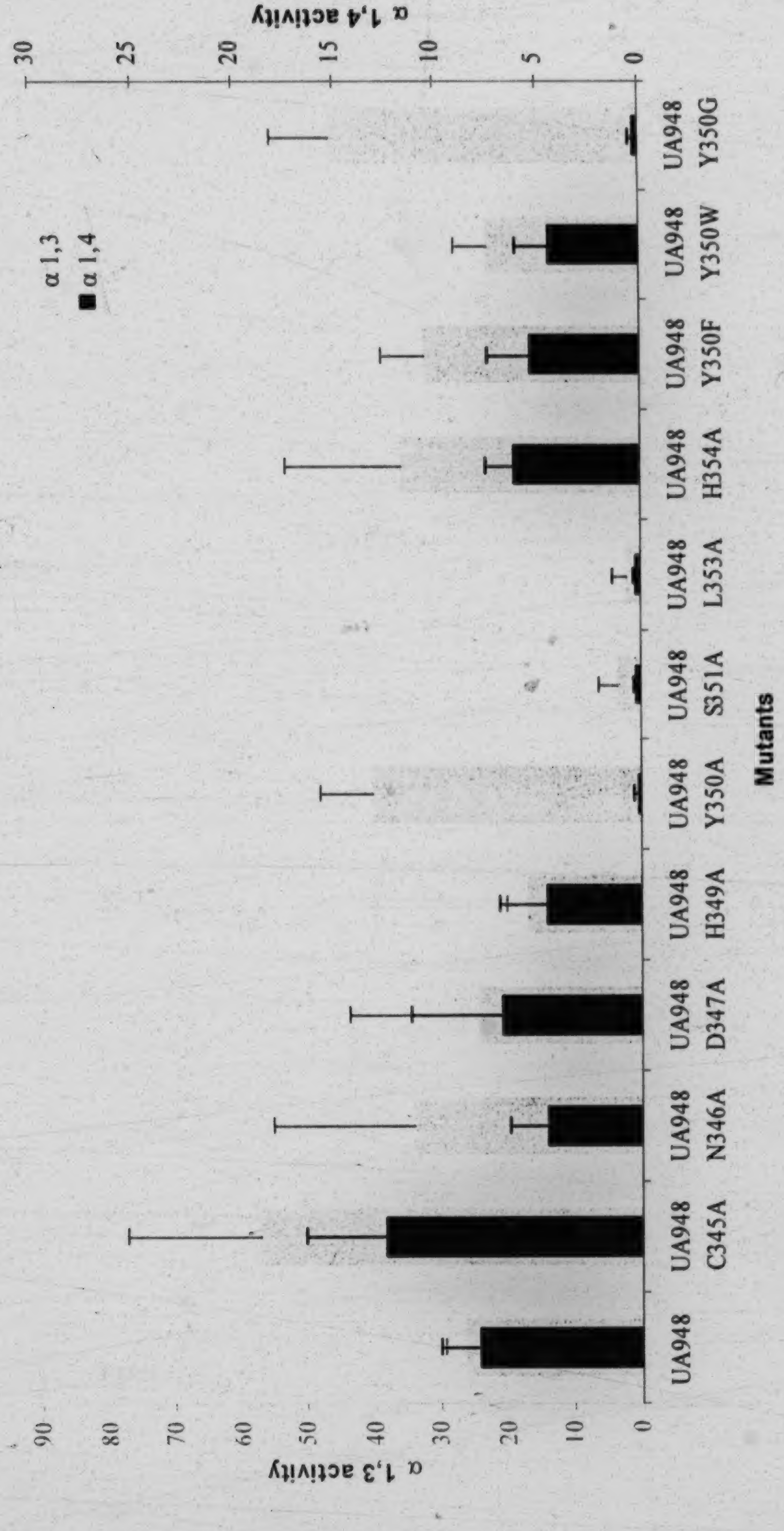
3.3.1 Substrate Specificity of UA948FucT Alanine Mutants

Alanine scanning site-directed mutagenesis was performed to identify which amino acids within ^{345}CNDAHYSALH^{354} of UA948FucT are required for α1,4 activity. The α1,3 and α1,4 activities of all mutants with single amino acid substitutions were determined with Type II-R and Type I-R substrates at 1.8 mM and 7.5 mM, respectively (Fig. 3-1). Mutations with a significant effect on enzyme activity clustered in residues 350 to 353. Alanine substitutions of Ser^{351} and Leu^{353} drastically decreased both α1,3 and α1,4 activities, with the α1,4/α1,3 ratio reduced to 22.6% and 26.3% of that of WT, respectively. Notably, alanine substitution of the only aromatic residue in the loop sequence, Tyr^{350}, had the most dramatic effect. It reduced α1,4 activity almost 58 fold without decreasing α1,3 activity (Fig. 3-1). The α1,4/α1,3 ratio of Tyr^{350}→Ala mutant is 1.8% of that of WT. This indicates that Tyr^{350} is specifically required for α1,4 activity in UA948FucT.

3.3.2 Substrate Specificity of UA948FucT and 11639FucT Tyrosine and Phenylalanine Mutants

The Tyr^{350}→Ala mutation removes both the aromatic ring and the hydroxyl group of Tyr. To determine the contribution of each group to Type I acceptor specificity in UA948FucT, Tyr^{350} was mutated to Phe, Trp and Gly. These mutations did not cause any decrease in α1,3 activity. However, the α1,4 activities were reduced to 66.9%, 55.6% and 3.1% of the WT level and their α1,4/α1,3 ratios were at 55.6%, 65.2% and 1.8% of the

Fig. 3-1. Enzyme specific activities of wild type and mutant UA948FucT. Enzyme specific activities (mU mg^{-1}) for α1,3-FucT (gray bars, left Y-axis) and α1,4-FucT (black bars, right Y-axis) activities of UA948FucT and each single amino acid mutant were standardized by FucT expression level. Each value represents the average of three or four determinations with standard deviations indicated.


α 1,3 activity
0
10
20
30
40
50
60
70
80
90
α 1,4 activity
0
5
10
15
20
25
30
α 1,3
α 1,4
UA948
UA948 C345A
UA948 N346A
UA948 D347A
UA948 H349A
UA948 Y350A
UA948 S351A
UA948 L353A
UA948 H354A
UA948 Y350F
UA948 Y350W
UA948 Y350G
Mutants

WT level, respectively (Fig.3-1). The data demonstrated that the aromatic ring structure present in Tyr, Phe and Trp but absent in Ala and Gly is essential for α1,4 activity in UA948FucT, whereas the hydroxyl group in Tyr^{350} is optimal but not absolutely required.

The hypervariable region ^{347}DNPFIFC^{353} in 11639FucT contains two Phe residues, but it shares virtually no sequence homology with the hypervariable loop of UA948FucT. It is not clear which Phe, if any, could be aligned with Tyr^{350} in UA948FucT. Mutants 11639FucT Phe^{350}→Tyr and Phe^{352}→Tyr were constructed to determine if the addition of a hydroxyl group would improve α1,4 activity. In addition, to determine whether or not the presence of an extra bulky aromatic residue in the variable loop of 11639FucT (instead of a single aromatic amino acid in UA948FucT) is responsible for the poor α1,4 activity in 11639FucT, mutants 11639FucT Phe^{350}→Ala and Phe^{352}→Ala were also made.

In comparison to WT 11639FucT, mutants Phe^{350}→Tyr, Phe^{352}→Tyr and Phe^{350}→Ala possessed a similar level of α1,3 activity, whereas Phe^{352}→Ala displayed a reduced α1,3 activity at ~25% of WT level. None of the mutants Phe^{350}→Tyr, Phe^{352}→Tyr, Phe^{350}→Ala and Phe^{352}→Ala displayed any α1,4 activity in the standard radiochemical assay. However, when the cell lysates were concentrated 4-5 times and the Type I acceptor concentration in radiochemical assays was raised from 7.5 mM to 25 mM, trace amounts of α1,4 activity were detected from all four mutants as well as from WT 11639FucT at 0.01-0.16 mU mg^{-1}. Again, mutant Phe^{352}→Ala displayed the lowest α1,4 activity compared to WT and the other three mutants. Although α1,4 activity is just above the background level, it is detectable yet too weak for accurate quantification.

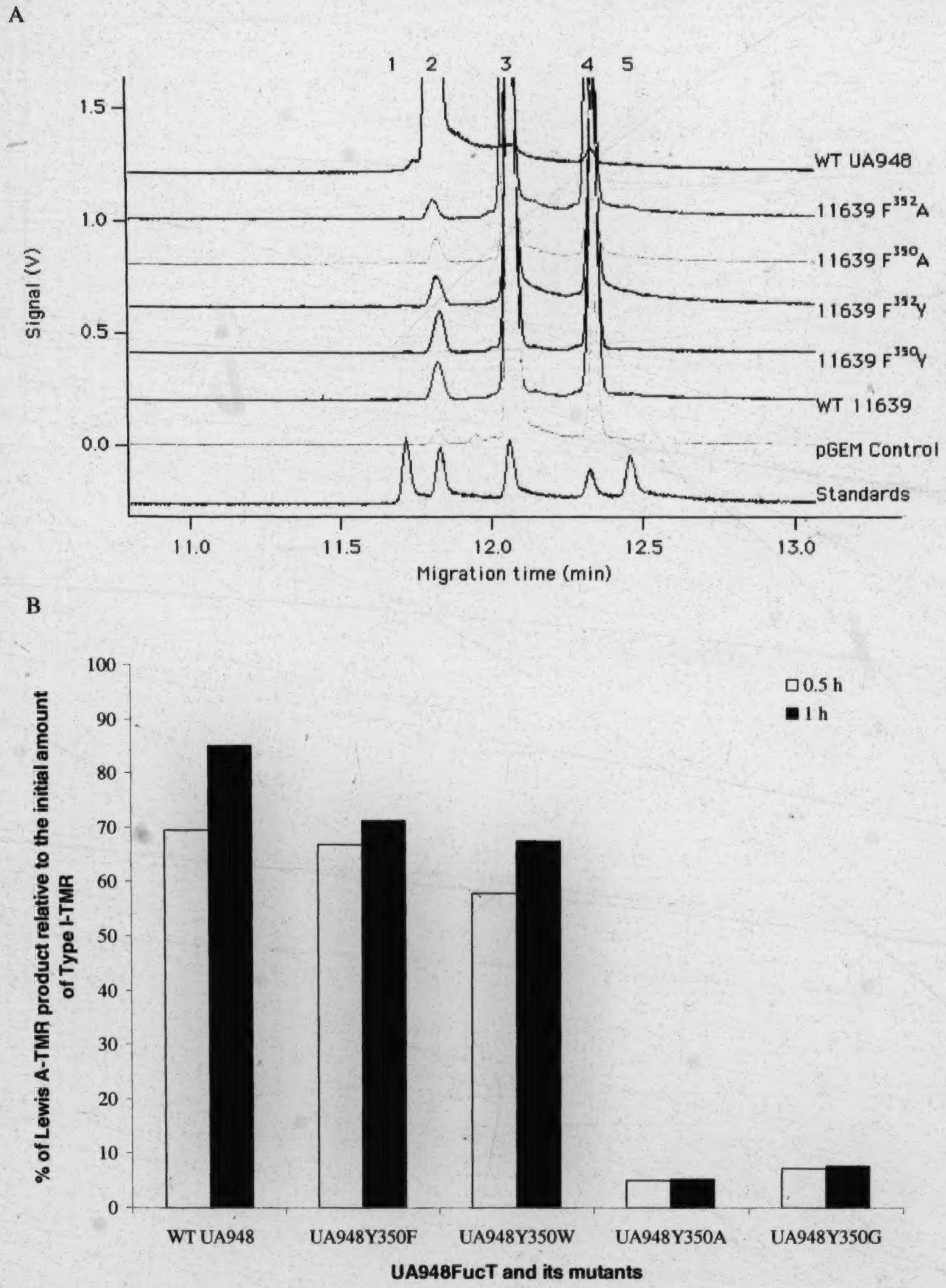
3.3.3 Capillary Electrophoresis Analysis

To measure the low level of α1,4 activity more precisely in WT 11639FucT and in its mutants Phe^{350}→Tyr, Phe^{352}→Tyr, Phe^{350}→Ala and Phe^{352}→Ala, capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was utilized with tetramethylrhodamine (TMR)-labeled Type I acceptor (Type I-TMR). CE-LIF is an ultra-sensitive analytical technique and can quantitate as few as 60 molecules of enzyme reaction product (Krylov *et al.*, 2000; Zhang *et al.*, 1995). The WT UA948FucT and its mutants were included as positive controls, and the pGEM vector without a *futA* insert was taken as a negative control.

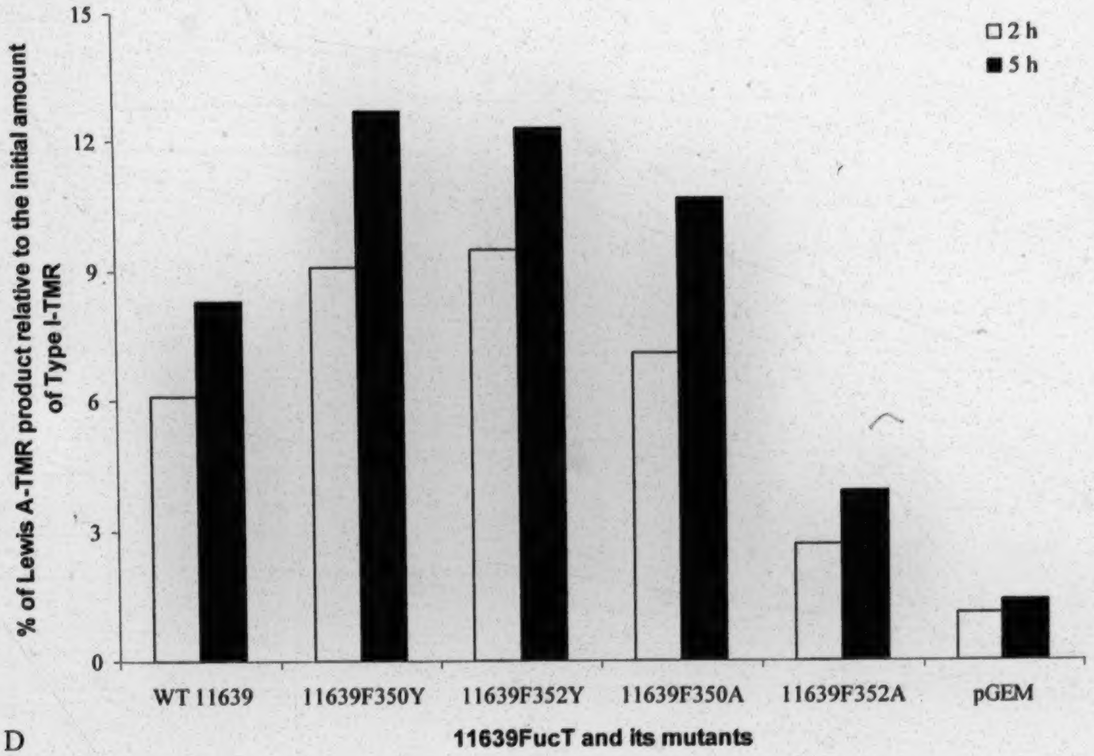
When the cell lysate of WT UA948FucT was incubated with 10 μM of Type I-TMR for 5 h, a new peak was produced as shown in the electropherogram (peak 2 in Fig. 3-2A). This new peak had the same migration time as a synthetic Lewis A-TMR used as running oligosaccharide standard (Fig. 3-2A, peak 2). The Lewis A-TMR production by WT UA948FucT and its mutants Tyr^{350}→Phe and Tyr^{352}→Trp was rapid with up to 58%-69.5% of Type I-TMR being converted at 30 min, in contrast to 5.1%-7.2% of Type I-TMR being converted by mutants Tyr^{350}→Ala and Tyr^{352}→Gly at 30 min (Fig. 3-2B). These CE results independently verified the radioactive assay data in Fig. 1. It is notable that a very small peak with a migration time similar to Lewis A-TMR was also observed in the pGEM vector control (Fig. 3-2A), but the α1,3/4-fucosidase treatment data clarified that this small peak did not represent Lewis A-TMR (see below).

The Lewis A peak was also detected in WT 11639FucT and its mutants Phe^{350}→Tyr, Phe^{352}→Tyr, Phe^{350}→Ala and Phe^{352}→Ala after 5 h incubation at 37°C, albeit at a very much lower level (Fig. 3-2A). Compared to UA948FucT, WT 11639FucT and its mutants catalyzed the fucose-transfer reaction very slowly. Only 3.9%-12.7% of Type I-TMR was

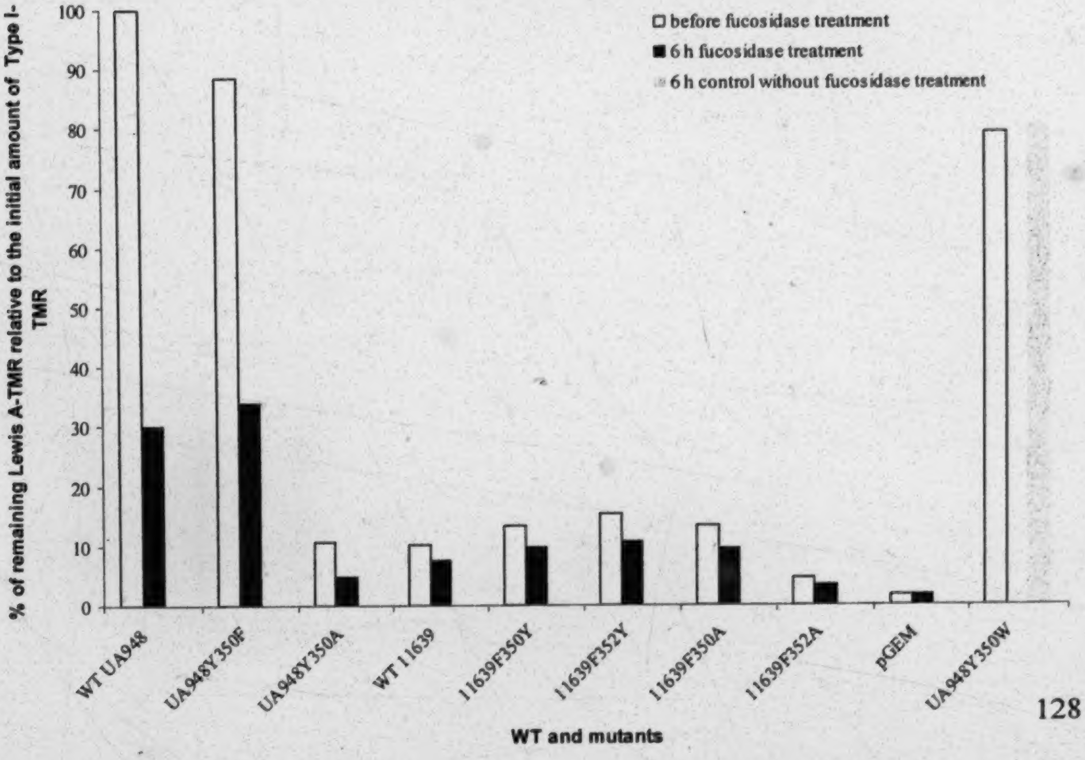
Fig. 3-2. Analysis of the reaction mixtures from wild type 11639FucT and UA948FucT, their mutants and pGEM vector control by capillary electrophoresis with laser-induced fluorescent detection. (A): Electropherogram showing the reaction products from a 5 h incubation containing substrate Type I-TMR (peak 3), donor GDP-Fuc and different *H. pylori* α1,3/4 FucTs or the pGEM vector control. Peak 2 corresponds to the Lewis A-TMR product. Peak 4 represents GlcNAc-TMR that was generated by the intrinsic β-galactosidase of host *E. coli* HMS174DE3 cells. The lowest gray trace is the separation of the five standard TMR oligosaccharides: Lewis B- (peak 1), Lewis A- (peak 2), Type I- (peak3), GlcNAc- (peak 4) and the linker arm-TMR (peak 5). **(B):** Quantitative analysis of the Lewis A-TMR production at 0.5 h and 1 h time points for WT UA948FucT and its mutants. **(C)**: Quantitative analysis of the Lewis A-TMR production at 2 h and 5 h time points for WT 11639FucT and its mutants. **(D):** Quantitative analysis of the percentage of Lewis A-TMR in the representative reaction mixtures relative to the initial amount of Type I-TMR before and after 6 h of α1,3/4-fucosidase treatment. Percentages values in **(B)**, **(C)** and **(D)** were standardized with the total protein concentration as well as the FucT expression level, which was detected by immuno-blotting.


A
1
2
3
4
5
1.5
1.0
0.5
0.0
Signal (V)
WT UA948
11639 F352A
11639 F350A
11639 F352Y
11639 F350Y
WT 11639
pGEM Control
Standards
11.0
11.5
12.0
12.5
13.0
Migration time (min)
B
100
90
80
70
60
50
40
30
20
10
0
% of Lewis A-TMR product relative to the initial amount of Type I-TMR
□ 0.5 h
■ 1 h
WT UA948
UA948Y350F
UA948Y350W
UA948Y350A
UA948Y350G
UA948FucT and its mutants

C


% of Lewis A-TMR product relative to the initial amount of Type I-TMR
15
12
9
6
3
0
□ 2 h
■ 5 h
WT 11639
11639F350Y
11639F352Y
11639F350A
11639F352A
pGEM
11639FucT and its mutants

D


% of remaining Lewis A-TMR relative to the initial amount of Type I-TMR
100
90
80
70
60
50
40
30
20
10
0
□ before fucosidase treatment
■ 6 h fucosidase treatment
▨ 6 h control without fucosidase treatment
WT UA948
UA948Y350F
UA948Y350A
WT 11639
11639F350Y
11639F352Y
11639F350A
11639F352A
pGEM
UA948Y350W
WT and mutants

converted to Lewis A-TMR by 5 h (Fig. 3-2C). Although this low activity may not play a role *in vivo*, it is of interest from a mechanistic perspective to see if either Phe residue mimics Tyr^{350} of UA948FucT. Compared to WT 11639FucT, both $\text{Phe}^{350}\rightarrow\text{Tyr}$ and $\text{Phe}^{352}\rightarrow\text{Tyr}$ mutations caused an approximately 50% increase in α1,4 activity. Mutant $\text{Phe}^{350}\rightarrow\text{Ala}$ exhibited a marginal increase in α1,4 activity, whereas $\text{Phe}^{352}\rightarrow\text{Ala}$ displayed a greater than 2-fold drop. Therefore, unlike Tyr^{350} in UA948FucT, neither Phe^{350} nor Phe^{352} in 11639FucT seems to be selectively associated with the α1,4 activity. The presence of two bulky aromatic residues in the hypervariable loop of 11639FucT is very unlikely the reason for poor α1,4 activity. It is noted that mutation $\text{Phe}^{352}\rightarrow\text{Ala}$, but not $\text{Phe}^{352}\rightarrow\text{Tyr}$, caused a reduction in both α1,3 and α1,4 activity, indicating that the aromatic nature of Phe^{352} may play an important role in fucose transfer to both Type I and Type II acceptors.

The synthesis of Lewis A-TMR by WT FucTs and the mutants was verified by subsequent α1,3/4-fucosidase treatment of the 5 h reaction mixture. α1,3/4-Fucosidase specifically removes the fucose group at the α1,3 or α1,4 linkage. The quantitative analysis of α1,3/4-fucosidase hydrolysis of Lewis A-TMR from WT FucTs, the representative mutants, and the pGEM control reactions are shown in Fig. 3-2d. The degradation of Lewis A-TMR to Type I-TMR was observed to various extents in WT FucT enzymes and their mutants but not for the pGEM vector control (Fig. 3-2D). The 5 h reaction mixture of mutant UA948FucT $\text{Tyr}^{350}\rightarrow\text{Trp}$ in sodium phosphate buffer without α1,3/4-fucosidase was also incubated. After 6 h, the Lewis A-TMR product remained at the same level (Fig. 3-2D), indicating that Lewis A-TMR is stable if not treated by α1,3/4-fucosidase. This confirms that the newly formed product (peak 2 in Fig.

3-2A) in WT FucTs and their mutants is indeed the fucosylated structure of Type I-TMR, whereas the small peak for the pGEM vector control (Fig. 3-2A) is due to an unrelated co-migrating substance of unknown identity.

3.3.4 Expression of WT and Mutant FucTs

The protein expression of WT and each mutant dreuvatuve if 11639FucT and UA948FucT with a His_6 tag at the C-terminus was determined by immunoblotting using an anti-penta-histidine monoclonal antibody. The expected molecular mass of 11639FucT and UA948FucT are 56.0 kDa and 54.6 kDa, respectively. Every UA948FucT mutant with either Ala or Phe substitution was expressed at a similar level to that of the WT (Fig. 3-3A), the same results were obtained with Trp and Gly substitution mutants (Fig3-3B). Mutants 11639FucT Phe^{350}→Tyr, Phe^{352}→Tyr, Phe^{350}→Ala and Phe^{352}→Ala were also expressed at a similar level to that of 11639FucT (Fig. 3-3C). These results indicate that point mutations did not cause significant changes in FucT enzyme expression levels.

3.3.5 Kinetic Parameters of Mutants

Kinetic parameters of WT FucTs and mutants from UA948FucT are shown in Table 3-2. Efforts were made to determine the kinetic parameters of WT 11639FucT and its mutants Phe^{350}→Tyr, Phe^{352}→Tyr, Phe^{350}→Ala and Phe^{352}→Ala for using Type I acceptor. Unfortunately, the K_m was very high, so that the kinetic curves were still linear when up to 25 mM of Type I acceptor was used (Fig. 3-4). As a result it was not possible to determine the kinetic parameters for these enzymes with confidence.

The acceptor kinetic data showed that the substitution of Tyr^{350} with Phe, Ala, Trp or Gly in UA948FucT did not modify the K_m to the Type II acceptor, whereas the V_{max} was either increased or retained at WT level (Table 3-2), suggesting that Tyr^{350} is not critical

Fig 3-3. Immuno-blot of wild type 11639FucT and UA948FucT and single amino acid mutants. Wild type and mutated FucTs with His_6-tag were expressed in *E. coli* HMS174DE3 cells with induction using 1mM of isopropyl-β-D-thiogalactopyranoside and growth at 30°C for 4 hours. The FucT proteins were detected with mouse anti-penta-histidine monoclonal antibody.

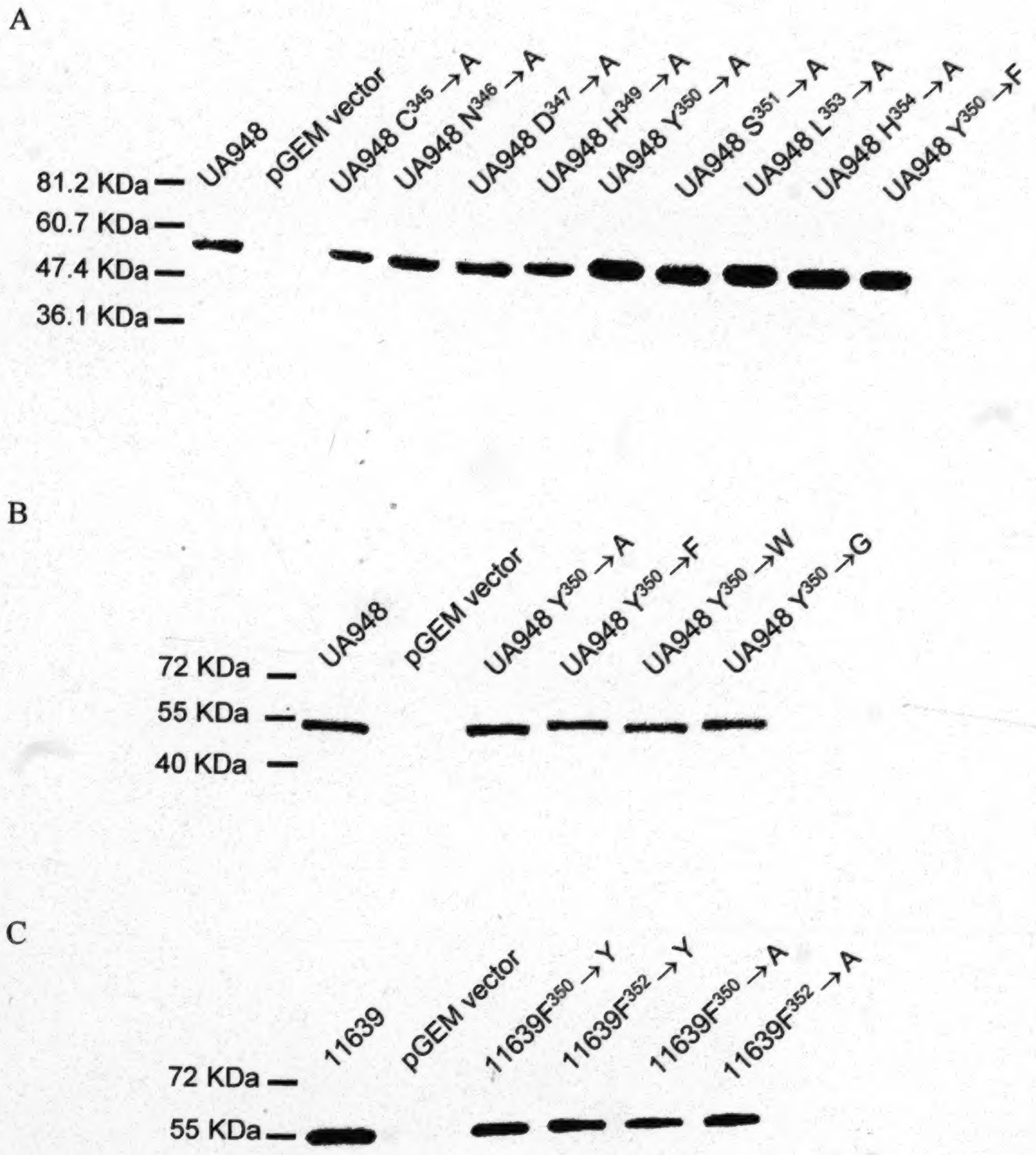

A
UA948
pGEM vector
UA948 C345 → A
UA948 N346 → A
UA948 D347 → A
UA948 H349 → A
UA948 Y350 → A
UA948 S351 → A
UA948 L353 → A
UA948 H354 → A
UA948 Y350 → F
81.2 KDa
60.7 KDa
47.4 KDa
36.1 KDa
B
UA948
pGEM vector
UA948 Y350 → A
UA948 Y350 → F
UA948 Y350 → W
UA948 Y350 → G
72 KDa
55 KDa
40 KDa
C
11639
pGEM vector
11639F350 → Y
11639F352 → Y
11639F350 → A
11639F352 → A
72 KDa
55 KDa

Table 3-2. Acceptor and donor kinetic parameters of wild type and single amino acid mutants. Acceptor kinetics were determined in cell crude extracts using 0.03-2 mM Type II-R, or 0.4-25 mM Type I-R with GDP-Fuc at 200 μM and GDP-^{3}[H]Fuc at 0.2 μM. Donor kinetics were determined using 3-200 μM GDP-fucose with Type II-R at 2 mM or Type I-R at 15 mM and GDP-^{3}[H]Fuc at 0.8 μM.

	Type II-R[b]			Type I-R[c]			K_m for GDP-Fuc (μM)	
WT/Mutants[a]	K_m (mM)	V_{max} [d]	V_{max}/K_m [e]	K_m (mM)	V_{max}	V_{max}/K_m [e]	Type II-R[b]	Type I-R[c]
11639	0.37 ± 0.03	11.8 ± 0.3	31.9	–[f]	–[f]	–[f]	44.7 ± 5.5	–[f]
UA948	1.5 ± 0.2	29.0 ± 1.8	19.3	13.4 ± 1.4	11.7 ± 0.6	0.87	56.0 ± 10.1	33.7 ± 7.8
UA948 Y^{350}→F	1.5 ± 0.2	45.0 ± 3.3	30	16.7 ± 1.4	12.1 ± 0.5	0.72	26.0 ± 2.2	15.2 ± 2.8
UA948 Y^{350}→A	1.7 ± 0.3	48.6 ± 4.9	28.5	24.4 ± 3.3	1.1 ± 0.1	0.05	26.4 ± 2.9	7.2 ± 2.0
UA948 Y^{350}→W	1.1 ± 0.1	29.0 ± 0.6	26.4	6.2 ± 0.4	7.7 ± 0.2	1.2	18.6 ± 2.5	11.8 ± 1.8
UA948 Y^{350}→G	1.8 ± 0.1	61.0 ± 1.9	33.9	19.6 ± 1.2	0.9 ± 0.03	0.05	21.1 ± 1.0	9.5 ± 2.5

[a] All FucTs possessed a His_6-tag at the C-terminus.

[b] Type II-R: Galβ1,4GlcNAc-$O(CH_2)_8CO_2CH_3$.

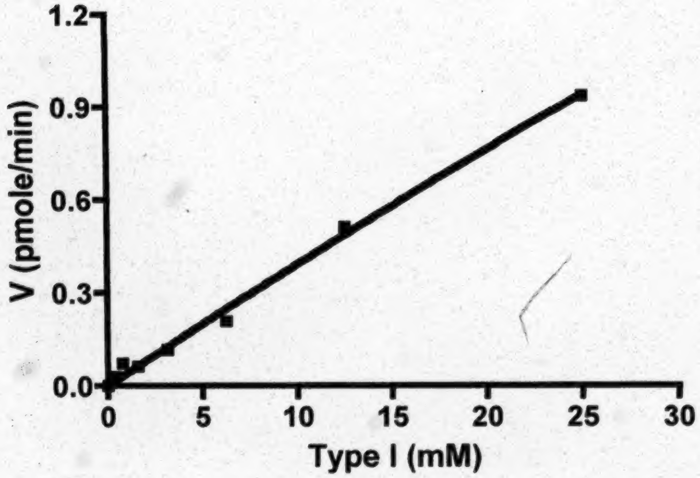
[c] Type I-R: Galβ1,3GlcNAc-$O(CH_2)_8CO_2CH_3$.

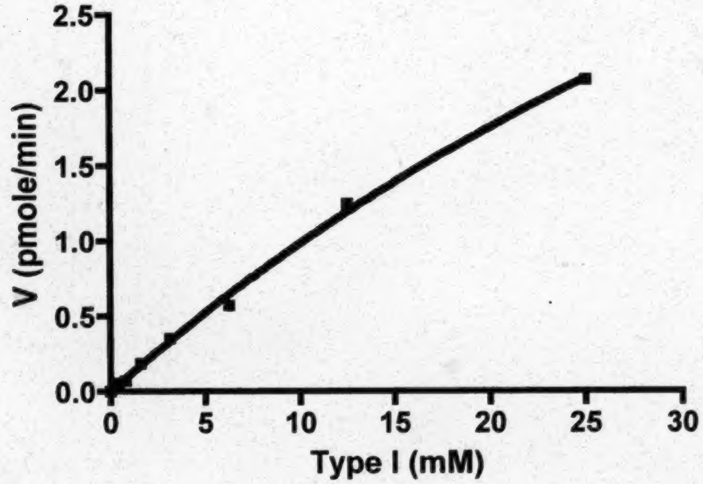
[e] V_{max}/K_m : mU mg^{-1} mM^{-1}.

[d] V_{max} (mU mg^{-1}) was standardized based on FucT expression quantified by Western Blot (Fig. 3-3).

-[f]: not determined due to the very low level of α1,4 activity thus the kinetic parameters could not be obtained with confidence.

Fig. 3-4. Fucose transfer velocity rate versus the concentration of Type I acceptor for mutants 11639Phe^{350}→Tyr (top) and 11639F^{352}→Tyr (bottom). Assays were performed with GDP-Fuc at 200 μM and GDP-^{3}[H]Fuc at 0.2 μM as described in Experimental Procedures. Non -linear regression was analyzed with Prism 4.0 software. The kinetic curves were still linear when up to 25 mM of Type I acceptor was used.


V (pmole/min)
1.2
0.9
0.6
0.3
0.0
0
5
10
15
20
25
30
Type I (mM)


V (pmole/min)
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
Type I (mM)

for Type II acceptor recognition. With Type I acceptor, mutant Tyr^{350}→Phe had similar K_m and V_{max} values when compared to WT UA948FucT. The K_m and V_{max} of mutant Tyr^{350}→Trp is half and two-thirds respectively of the WT level and its V_{max}/K_m is slightly higher than that of WT (Table 3-2). Thus the replacement of Tyr^{350} with Phe or Trp does not significantly affect α1,4 activity. In contrast, Tyr^{350}→Ala and Tyr^{350}→Gly showed a dramatic decrease in V_{max} with a moderate increase in K_m (Table 3-2), which resulted in a 17-fold decrease of V_{max}/K_m, indicating that the Ala and Gly substitutions not only severely impaired the catalytic rate but also decreased the Type I acceptor binding affinity. The kinetic data confirm that the aromatic nature of Tyr^{350} in UA948FucT is indispensable for α1,4 specificity.

In addition to the effects on acceptor binding, the K_m for the donor substrate GDP-fucose was modified by mutations of Tyr^{350}→Ala, Phe, Trp or Gly. All mutants displayed tighter binding to the donor than WT, with the apparent K_m value decreasing two-fold with Type II acceptor, and two-fold (Tyr^{350}→Phe and Tyr^{350}→Trp) or four-fold (Tyr^{350}→Ala and Tyr^{350}→Gly) with Type I acceptor. Such modification of donor binding caused by a single amino acid mutation is not totally unexpected (see below).

3.4 Discussion

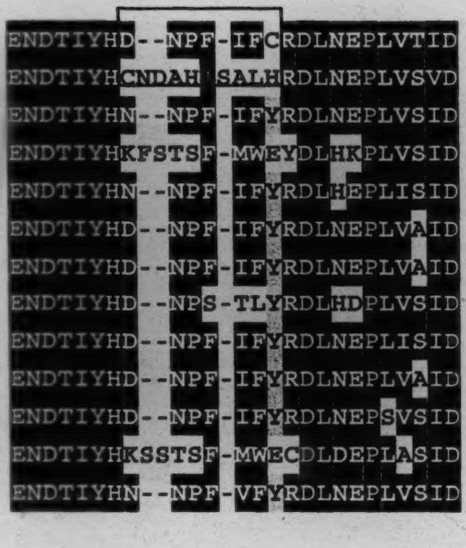
The previous domain swapping studies showed that the α1,4 specificity of UA948FucT is determined by the C-terminal hypervariable loop (Ma *et al.*, 2003). The current data demonstrate that within the loop, Tyr^{350} is the only residue that, if mutated to Ala, converts UA948FucT into an enzyme with predominantly α1,3 activity. In contrast, when Tyr^{350} is mutated to Phe or Trp, a significant level of α1,4 activity is retained, suggesting that the aromatic nature of residue 350 is required for α1,4 activity. Other residues in the loop, particularly Ser^{351} and Leu^{353} may play a role in catalysis as mutating them to Ala affects both α1,3 and α1,4 activity. A very similar hypervariable loop sequence (^{343}CDAHNYSALH^{352}) has recently been reported for the newly characterized *H. pylori* α1,3/4FucT from strain DMS6709. The underlined Tyr, Ser and Leu residues correspond to the critical residues of UA948FucT. Indeed, DMS6709FucT is primarily an α1,4 FucT with little α1,3 activity (Rabbani *et al.*, 2005).

In the previous work, a substantial level of α1,4 activity (11% of WT UA948FucT) was introduced into 11639FucT when its hypervariable loop was replaced with that of UA948FucT (Chimera 11639$^{347\text{CNDAHYSALH}}$) (Ma *et al.*, 2003). This is in contrast to the marginal augmentation of α1,4 activity that was currently observed when Phe^{350} or Phe^{352} in the loop of 11639FucT was mutated to Tyr. Apparently, the Tyr residue itself in the hypervariable loop of 11639FucT is not sufficient for conferring significant α1,4 activity. Some other features, as yet not recognized, within the loop in UA948FucT are certainly needed and perhaps the length of the loop is also critical. While the vital role of the C-terminal hypervariable loop is highlighted, particularly the aromatic nature of Tyr^{350}, in controlling the α1,4 specificity in UA948FucT, the fact that other residues also contribute to conferring the α1,4 activity should not be neglected, largely the 110 residues at the N-terminus, as concluded from previous domain swapping study (Ma *et al.*, 2003).

The hypervariable loop of 11639FucT contains two aromatic residues (Phe^{350} and Phe^{352}). The CE-LIF data show that neither Phe^{350} nor Phe^{352} is specifically associated with the α1,4 activity, but Phe^{352} seems to be crucial for both α1,3 and α1,4 activity. No matter if one or two aromatic residues (Phe or Tyr) is in the hypervariable loop, 11639FucT lacks a substantial level of α1,4 activity. This indicates that, unlike Tyr^{350} in UA948FucT, the aromatic residue at neither 350 nor 352 in 11639FucT seems to be located at the favorable position for fucose transfer to the Type I acceptor. In addition, 11639FucT also lacks the other determinants that contribute to a significant level of α1,4 activity in UA948FucT. As discussed above, such determinants may include the unique features in the hypervariable loop and the N-terminal 110 amino acids. Similarly, no α1,4 activity was obtained in bovine FucT when its Arg residue was replaced by Trp, which has been shown to be the single residue that confers the α1,4 specificity in human FucT III (Dupuy *et al.*, 1999).

UA948FucT is to date the only *H. pylori* enzyme that has been reported to possess substantial α1,4 activity. Low α1,4 activity has been demonstrated for both UA1111FucTa and UA1111FucTb (Rasko *et al.*, 2000a). 11637FucTb was shown to only use Type II but not Type I as an acceptor thus it is an α1,3 FucT, even though it can transfer fucose to Type I tetrasaccharide (Galβ1,3GlcNAcβ1,3Galβ1,4Glc) but the fucose was predominantly transferred to the glucose residue (Martin *et al.*, 1997). FucTs from strain 26695, J99, Sydney SS1, UA1182 and UA802 are predicted to have exclusive α1,3 activity, as Type II Lewis antigens, but not Type I Lewis antigens, were detected on the LPS of these strains by ELISA, immunoblot, NMR spectroscopy or fast atom bombardment-mass spectrometry (Monteiro *et al.*, 2000; Wang *et al.*, 1999b). The sequence alignment of the C-terminal segment of these *H. pylori* FucTs (Rasko, 2000) shows that the hypervariable loop region of UA948FucT differs considerably from all other *H. pylori* FucTs (Fig. 3-5). Nine *H. pylori* FucTs, including 11639FucT and UA1111FucTa, possess an almost identical hypervariable loop. Austra244 FucT contains

Fig. 3-5. Alignment of C-terminal hypervariable loop region of 13 *H. pylori* α1,3/4 FucT enzymes. Sequence alignments were performed using ClustalW from the LaserGene99 software DNASTAR. Identical residues are on a black background. Highly conserved residues have a dark gray background, while partially conserved residues are shown on a light gray background. Numbering at the end of each line refers to the position in each sequence. The α1,3 or α1,4 activity of each FucT is shown in the right column, and has either been determined by radiochemical assays, or predicted based on the Lewis antigen expression pattern, or is unknown. The framed amino acids are the residues that have been identified as being responsible for acceptor specificity in 11639FucT and UA948FucT (Ma *et al.*, 2003). Of note, due to the highly divergent sequence of the hypervariable loops of 11639FucT and UA948FucT, one cannot assume that either of the two Phe residues in the 11639FucT loop is aligned with Tyr^{350} of UA948FucT.

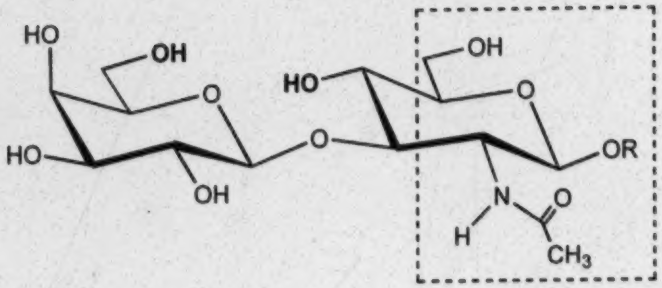


H. pylori FucT		Coordinate	FucT activity
11639FucTa	ENDTIYHD--NPF-IFCRDLNEPLVTID	364	α1,3 & low α1,4
UA948FucTa	ENDTIYHCNDAH-SALHRDLNEPLVSVD	365	α1,3 and α1,4
11637FucTb	ENDTIYHN--NPF-IFYRDLNEPLVSID	364	α1,3
26695FucTa	ENDTIYHKFSTSF-MWEYDLHKPLVSID	367	Predicted α1,3
26695FucTb	ENDTIYHN--NPF-IFYRDLHEPLISID	396	Predicted α1,3
J99FucTa	ENDTIYHD--NPF-IFYRDLNEPLVAID	364	Predicted α1,3
J99FutTb	ENDTIYHD--NPF-IFYRDLNEPLVAID	364	Predicted α1,3
Austra244	ENDTIYHD--NPS-TLYRDLHDPLVSID	363	Unknown
UA1182	ENDTIYHD--NPF-IFYRDLNEPLISID	364	Predicted α1,3
SydneySS1	ENDTIYHD--NPF-IFYRDLNEPLVAID	361	Predicted α1,3
UA1111FucTa	ENDTIYHD--NPF-IFYRDLNEPSVSID	363	low α1,3 & α1,4
UA1111FucTb	ENDTIYHKSSTSF-MWECDLDEPLASID	366	low α1,3 & α1,4
UA802FucTa	ENDTIYHN--NPF-VFYRDLNEPLVSID	391	Predicted α1,3

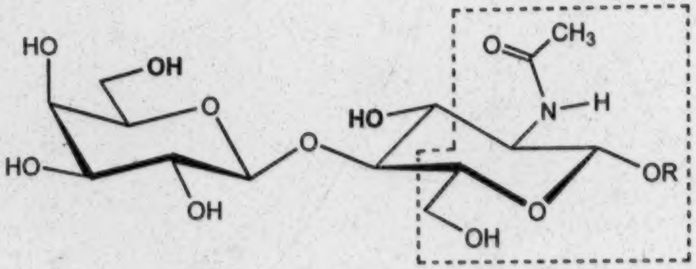
a similar loop but with three distinctive amino acids. The loop of 26695FucTa and UA1111FucTb is two amino acids longer and six residues are divergent. In the attempt to measure the α1,4 activity in 11639FucT mutants, it is noted that even 11639FucT, which was previously considered as an α1,3 FucT (Ge *et al.*, 1997; Ma *et al.*, 2003), displays a very weak but detectable α1,4 activity when the sensitivity of the assay is increased. As many *H. pylori* FucT enzymes contain a loop sequence that is identical to that of either 11639FucTa, UA1111FucTa or UA1111FucTb, it is possible that weak α1,4 activity is present in all these *H. pylori* FucTs, but the activity level is too low to be detected by less-sensitive assays. The Lewis A structure generated on the LPS in these strains most likely is too scarce to be biologically significant. Nevertheless, the observation would be of interest for the evolution of the FucT family. It is speculated that the acquisition of a significant α1,4 activity in some *H. pylori* FucTs would have been facilitated by the presence of an intrinsically weak α1,4 activity in this family.

The promiscuity of FucT enzymes to act on both Type I and Type II acceptors might result from the stereochemical similarity of these two isomers. The structural differences between Type I and Type II are largely confined to the orientation of the *N*-acetamido and 6-CH_2OH groups in GlcNAc moiety. In Type II substrates, the *N*-acetamido group is on the same side as the fucose addition site (OH-3 of GlcNAc), whereas in Type I substrate this position is occupied by 6-CH_2OH (Biswas and Rao, 1980; Khare DP, 1985; Lemieux, 1980) (Fig. 3-6). When oligosaccharide (including donor and acceptor substrate) binds to a glycosyltransferase during the catalytic reaction, the oligosaccharide can be distorted (Bourne *et al.*, 1992; Bush *et al.*, 1999; Homans, 1993). This was confirmed in several glycosyltransferases with resolved crystal structures as reviewed

Fig. 3-6. Structures of Type I and Type II acceptors. The hydroxyl groups required for recognition by human α1,3/4 FucTs in Type I and Type II structures are in bold. Structural difference between Type I and Type II acceptors are OH-1, CH_2OH-6 and the N-acetamido groups in the GlcNAc moiety that are framed with the dotted lines.



Type I (Galβ1,3GlcNAc)



Type II (Galβ1,4GlcNAc)

previously (Qasba *et al.*, 2005). Using a panel of monodeoxygenated Type I and Type II acceptor substrates, the 6-OH of the galactose moiety in both Type I and Type II acceptors and the reactive hydroxyl group (the OH-4 and OH-3 of GlcNAc in Type I and Type II, respectively) were found to be essential for the recognition by human FucT III, IV, V (de Vries *et al.*, 1995), VI (De Vries *et al.*, 1997), and human milk α1,3 and α1,3/4 FucTs (Du and Hindsgaul, 1996; Gosselin and Palcic, 1996). This suggests that Type I and Type II acceptors, when they bind to human α1,3 and α1,3/4 FucTs, most likely adopt a conformation so that the OH-4 of GlcNAc in Type I acceptor is placed in the same position relative to the 6-OH of the galactose moiety as is the OH-3 of GlcNAc in Type II acceptor. To achieve this, the orientation of the GlcNAc in Type I structures needs to be rotated by 180° relative to the galactose moiety, with the nonpolar side of the pyranose ring facing the opposite direction (Fig. 3-6). This hypothesis is strengthened by NMR studies of Lewis X and Lewis A trisaccharides, where the fucose and galactose in both compounds occupy very similar relative positions with the major difference being the 180° flip of the GlcNAc moiety (Azurmendi *et al.*, 2002). Thus, to be able to use Type I as an acceptor, FucT enzymes need to support the binding of the GlcNAc in the inverted orientation and to accommodate the reversed orientation of the N-acetamido and 6-CH_2OH groups with the former being bulkier and more hydrophobic. The same situation may apply to *H. pylori* FucTs as the 6-OH of galactose was also found to be critical for the binding of Type I and Type II acceptors to 11639FucT and UA948FucT (unpublished data). Previous work has shown that in mammalian α1,3/4 FucTs, an aromatic residue (Trp) is responsible for Type I acceptor specificity, even though it is located at the N-terminal hypervariable region (Dupuy *et al.*, 1999; Dupuy *et al.*, 2004).

One could speculate therefore that mammalian and *H. pylori* FucTs that possess α1,4 activity may require a crucial aromatic residue to interact with the nonpolar face of the Type I acceptor GlcNAc moiety in its inverted orientation to assure efficient fucose transfer.

To gain greater insight in the potential mechanism of substrate specificity, the kinetic parameters for four Tyr^{350} mutants of UA948FucT were characterized (Table 3-2). It was noted that mutations of Tyr^{350} to non-aromatic residues generated a moderate increase of K_m value but a rather dramatic reduction of $V_{\max}$, implying that the aromatic side chain of residue 350 in UA948FucT is possibly more related to catalytic efficiency than acceptor affinity. All mutations, on the other hand, also caused a two- to four- fold decrease of donor K_m values. The finding that a mutation causes modifications of both acceptor and donor binding affinity is not entirely unexpected. The analysis of several glycosyltransferase crystal structures has revealed a common theme for their kinetic mechanisms, with the sugar-nucleotide donor binding to the enzyme first followed by the acceptor (Qasba *et al.*, 2005). The donor binding event induces a marked conformational change of the enzyme in one or two flexible loops from an open to a closed conformation. Such change allows the loops to act as a lid to cover the bound donor and to create the binding sites for the acceptor. Thus the non-reducing end of the acceptor is held in position close to the sugar donor in the active sites prior to the start of the transfer reaction. After the formation of the new glycosidic bond, the oligosaccharide product is released first followed by the release of the nucleotide portion of the donor and eventually the flexible loops return to their original conformation to start a new catalytic cycle (Qasba *et al.*, 2005). Product inhibition studies for human FucT V suggest that this enzyme also follows an ordered sequential bi-bi mechanism (Qiao *et al.*, 1996) and it is likely that the *H. pylori* FucTs do the same. It is tempting to speculate that the hypervariable loop of *H. pylori* FucTs corresponds to a similar flexible active site loop that interacts with both donor and acceptor substrates. However, alternative mechanisms are possible and a crystal structure is ultimately needed to reveal the structural basis for enzyme activity and specificity. A better understanding of substrate specificity of *H. pylori* FucTs may enable to engineer new enzymes with improved specificity for either Type I or Type II substrates. Such enzymes could be used to synthesize biologically important carbohydrates that contain Lewis structures and might be potential pharmaceuticals in the prevention of bacterial or viral infection, in the neutralization of toxins, and in immunotherapy for cancer (Dwek, 1996; Endo and Koizumi, 2000; Guo and Wang, 1997; Johnson, 1999; Watt *et al.*, 1997).

3.5. Acknowledgments

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Purification, Kinetic Characterization and Mapping of the Minimal Catalytic Domain and Key Polar Groups for *Helicobacter pylori* α1,3/4 Fucosyltransferases

A version of this work has been submitted to *J. Biol. Chem.* for publication.[1,2]

Ma, B. Audette, GF. Lin, S. Palcic, MM, Hazes B and Taylor DE. Purification, kinetic characterization and mapping of the minimal catalytic domain and key polar groups for *Helicobacter pylori* α1,3/4 fucosyltransferases (currently under revision).

[1]: FucT purification by IMAC and AIEX was performed by Gerald F. Audette.

[2]: Enzymatic synthesis of Lewis X and Lewis A was performed by Shuangjun Lin.

4.1 Introduction

Fucosyltransferases (FucTs) catalyze the transfer of the L-fucose moiety from guanosine diphosphate β-L-fucose (GDP-Fuc) to glycoconjugate acceptors in an α1,2-, α1,3-, α1,4-, or α1,6 linkage. Extensive characterizations have been carried out on mammalian α1,3/4 FucTs with respect to the domain and amino acids responsible for donor binding (Holmes *et al.*, 1995; Holmes *et al.*, 2000; Jost *et al.*, 2005; Kimura *et al.*, 1997; Sherwood *et al.*, 1998; Sherwood *et al.*, 2002; Shinoda *et al.*, 1998), acceptor specificity (De Vries *et al.*, 1997; de Vries *et al.*, 2001b; Dupuy *et al.*, 1999; Dupuy *et al.*, 2004; Jost *et al.*, 2005; Legault *et al.*, 1995; Nguyen *et al.*, 1998; Sherwood *et al.*, 2002; Vo *et al.*, 1998), the key polar groups of acceptors that are essential for enzyme recognition (de Vries *et al.*, 1995; De Vries *et al.*, 1997; Du and Hindsgaul, 1996; Gosselin and Palcic, 1996), as well as their catalytic mechanism (Murray *et al.*, 1996; Murray *et al.*, 1997; Qiao *et al.*, 1996). In contrast, the structure-function study of prokaryotic α1,3/4 FucTs is not as far advanced (Ge *et al.*, 1997; Ma *et al.*, 2003; Martin *et al.*, 1997; Rabbani *et al.*, 2005; Rasko *et al.*, 2000b).

Both α1,2 and α1,3/4 FucTs have been identified and characterized in *H. pylori* (Ge *et al.*, 1997; Martin *et al.*, 1997; Rabbani *et al.*, 2005; Rasko *et al.*, 2000a; Rasko *et al.*, 2000b; Wang *et al.*, 1999a; Wang *et al.*, 1999b). These enzymes add fucose to the O antigen of *H. pylori* LPS to produce Lewis antigen structures, which is regulated by phase variation at rate of approximately 0.2-0.5% (Appelmelk *et al.*, 1998; Appelmelk *et al.*, 1999; Appelmelk *et al.*, 2000). Increasing evidence has shown that Lewis antigens are not an absolute prerequisite for *H. pylori* colonization and adhesion to the gastric epithelial cells. Instead, they play only a minor role in both processes (Altman *et al.*, 2003; Guruge *et al.*, 1998; Suresh *et al.*, 2000; Takata *et al.*, 2002). Nevertheless, expression of Lewis antigens on *H. pylori* LPS is often associated with the severe gastric pathology (Eaton *et al.*, 2004; Monteiro *et al.*, 2001; Rasko *et al.*, 2001). It has also been reported that Lewis antigens bind to dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (Appelmelk *et al.*, 2003; Bergman *et al.*, 2004) on dendritic cells modifying the host immune response (Bergman *et al.*, 2004).

Sequence alignments of mammalian and *H. pylori* α1,3/4 FucTs shows that they share very weak sequence homology (Rasko, 2000; Rasko *et al.*, 2000b) limited to two small regions designated as α1,3 FucT motifs (Martin *et al.*, 1997). These motifs have been suggested to be most likely involved in the binding of donor or metal ions (i.e. manganese) (Martin *et al.*, 1997; Oriol *et al.*, 1999). Mammalian α1,3/4 FucTs contain a short cytoplasmic N-terminal tail, a transmembrane segment followed by a hypervariable stem domain and a C-terminal catalytic domain. The transmembrane domain attaches mammalian FucTs at the Golgi membrane with the N-terminal tail facing the cytosol and the C-terminal catalytic domain exposed to the Golgi lumen (Fig. 2-5A). In vitro enzyme assay data showed that for human FucT III and V, deletion of the N-terminal 61 and 75 amino acids did not lead to significant loss of activity, whereas removal of one or more amino acids at the C-terminus in FucT V completely abolished enzyme activity (Xu *et al.*, 1996).

In contrast, *H. pylori* α1,3/4 FucTs lack the N-terminal cytosolic tail, the transmembrane domain and a large portion of the N-terminal stem region (Ge *et al.*, 1997; Martin *et al.*, 1997; Rasko *et al.*, 2000b). Instead, downstream of the catalytic domain, they contain a short hypervariable region followed by 2-10 heptad repeats (Ge *et* *al.*, 1997; Martin *et al.*, 1997; Rasko, 2000; Rasko *et al.*, 2000b) and a short segment, which is rich in hydrophobic and positive residues that meet the requirements to form two amphipathic α-helices (Fig. 2-5B) (Ma *et al.*, 2003). These helices are proposed to function as a membrane anchor with the hydrophobic face embedded in the membrane and the positive charges interacting with phospholipid head groups (Fig. 2-5B) (Ma *et al.*, 2003). In most *H. pylori* α1,3/4 FucTs, the heptad repeat consists of the amino acids DDLRVNY (Rasko, 2000; Rasko *et al.*, 2000b). 11639FucT contains 10 heptad repeats with the conserved sequence DDLR(V/I)NY, whereas UA948FucT has 5 internal ones with this consensus sequence but the first and the last two heptad repeats consist of the amino acid sequence DDLRRDH (Fig. 2-2) (Rasko, 2000; Rasko *et al.*, 2000b). It was suggested that the heptad repeat region contains a leucine-zipper like motif responsible for dimerization, which might be essential for enzyme function (Ge *et al.*, 1997; Martin *et al.*, 1997; Rasko *et al.*, 2000b). As a result, the C-terminal amphipathic α-helices and the preceding heptad repeat region in *H. pylori* α1,3/4 FucTs are functionally equivalent to the N-terminal transmembrane domain and the stem region of mammalian counterparts, respectively, but at their N-terminus (Fig. 2-5) (Ma *et al.*, 2003). For *H. pylori* FucT from strain NCTC11639, deletion of the C-terminal 115 amino acids, which contain the entire heptad repeat region and its downstream C-terminal tail including the putative amphipathic α-helices, completely eliminated enzyme activity (Ge *et al.*, 1997).

Both mammalian and *H. pylori* α1,3/4 FucTs display distinct Type I and Type II substrate preferences. Domain swapping and site-directed mutagenesis studies show that the Type I acceptor specificity is determined by their hypervariable regions, which for mammalian and *H. pylori* FucTs is at the opposite ends of their catalytic domains

(Legault *et al.*, 1995; Ma *et al.*, 2003; Nguyen *et al.*, 1998). In particular, a single aromatic residue is responsible for specificity (Dupuy *et al.*, 1999; Dupuy *et al.*, 2004; Ma *et al.*, 2005). For human α1,3/4 FucTs, the essential elements of acceptors required for recognition were also mapped. This was achieved by employing a panel of modified Type I- and Type II-series acceptors. For human FucT III, IV, V (de Vries *et al.*, 1995) and FucT VI (De Vries *et al.*, 1997), every hydroxyl group except the 6-OH of galactose and 3-OH or 4-OH of GlcNAc in Type II and Type I acceptor, respectively, tolerated modification. Consequently the 6-OH of galactose and the reactive hydroxyl groups of Type II and Type I acceptors were concluded to be the key polar groups essential for recognition by human α1,3/4 FucTs (de Vries *et al.*, 1995; De Vries *et al.*, 1997; Du and Hindsgaul, 1996; Gosselin and Palcic, 1996). The acceptor specificity of using modified oligosaccharide acceptors has also been carried out on *H. pylori* α1,3/4 FucTs, but a full characterization of the key polar groups has not been completed. The FucT from strain NCTC11637 is able to use 3'-sialyl-Type II but not 6'-sialyl-Type II as an acceptor (Martin *et al.*, 1997). Strain DMS6709 FucT is primarily an α1,4 FucT, and 3'-sialyl-Type I is an excellent acceptor (Rabbani *et al.*, 2005). This suggests that the hydroxyl at C-3 of galactose is not required for recognition by *H. pylori* α1,3/4 FucTs.

At present, no crystal structure is available for any member of the FucT family yet a crystal structure would provide excellent insight into enzyme-substrate binding and the catalytic mechanism. The first challenge to achieving this goal is to isolate large quantities of soluble and stable FucT. In the current study, truncations were made at both the N- and C-terminus of *H. pylori* α1,3/4 FucTs to delineate the minimal catalytic domain. Since removal of the C-terminal putative amphipathic α-helices increased both protein expression and solubility yet did not significantly reduce the specific enzyme activity, the truncated forms of *H. pylori* α1,3/4 FucTs were successfully purified at yield of milligrams per liter. These purified enzyme preparations were used to determine the kinetic parameters, key polar groups of acceptors essential for enzyme recognition and the potential of using these enzymes for enzymatic synthesis of Lewis X and Lewis A structures.

4.2 Experimental Procedures

4.2.1 Materials

Primers for constructing the truncated FucTs (ΔFucTs) were synthesized by Invitrogen Canada Co. (Burlington, ON). Pwo DNA polymerase and alkaline phosphatase were purchased from Roche (Mississauga, Ontario). pGEM-T vector was obtained from Promega Co. (Madison, WI). Type II-R (Galβ1,4GlcNAc-O-$(CH_2)_8CO_2CH_3$) **(1)**, Type I-R (Galβ1,3GlcNAc-O-$(CH_2)_8CO_2CH_3$) **(13)** and Type II-series **(2)-(12)** and Type I-series **(14)-(18)** modified compounds were kindly provided by Dr. Ole Hindsgaul. Anti-penta-histidine monoclonal antibody, plasmid mini-preparation and midi-preparation kits were purchased from QIAGEN Co. (Mississauga, ON). GDP-Fuc and horse-radish peroxidase conjugated goat anti-mouse IgG were from Sigma Chemical Co. (St Louis, MO). GDP-^{3}[H]Fuc (0.1 mCi ml^{-1}, 17.3 Ci $mmol^{-1}$) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). C_{18} reverse-phase cartridges were purchased from Waters (Milford, MA). The BCA protein assay kit and Bio-Rad protein assay kit were obtained from Pierce Biotech Inc. (Rockford, IL) and Bio-Rad (Mississauga, ON), respectively. Coomassie blue was purchased from Bio-Rad (Mississauga, ON). Nitrocellulose membrane was obtained from Micron Separation Inc.

(Westboro, MA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Biosciences Corp. (Buckinghamshire, England). BioMax MR film was obtained from Eastman Kodak Co. (Rochester, NY). HisTrap chelating HP Ni-NTA columns and Source 30Q Anion Exchange columns were obtained from GE Healthcare (Piscataway, NJ).

4.2.2 Cloning the ΔFucTs

WT *futA* gene of 11639FucT or UA948FucT was cloned into pGEM-T vector with a His_6-tag fused at the C-terminal end (Ma *et al.*, 2003). Plasmid DNA harboring the *futA* gene from strain NCTC11639 and UA948 were used as DNA template for PCR reactions. Primers for the FucTs from 11639 or UA948 were designed to delete different lengths of sequence at either the 5' or 3' end. Deletion of the entire (n) or n-1 heptad repeats but retaining the downstream C-terminal tail was achieved by primer over-extension with two steps of sequential PCR (Sambrook and Russel, 2001). The sequences of the primers are given in Table 4-1. The codons for the six histidine residues were included in the antisense primers when amplifying each truncated FucT. The PCR products were cloned into the A/T cloning vector pGEM-T under the control of the T7 promoter. Cloned truncated *futA* genes were sequenced to ensure that no mutation had occurred during their construction.

4.2.3 Expression of ΔFucTs

Full-length and each ΔFucT were expressed in *E. coli* HMS174DE3 cells as described previously (Ma *et al.*, 2003).

4.2.4 Separation of Soluble and Membrane Fractions of WT and C-terminal Truncated FucTs

Table 4-1. Sequences of primers used to construct truncated FucTs. The primer names with BM# and descriptive names are given in the first and second column, respectively. The descriptive primer names begin with the *H. pylori* strain to be truncated, "11639", "UA948" or both if they share the same sequence and end with an "F" or "R" to designate the forward (sense) and reverse (anti-sense), primer, respectively. A Shine-Dalgarno sequence was included in sense primers of 11639F, UA948F, $11639^{10\text{-}447}$F and $UA948^{10\text{-}434}$F and is in bold italic. The start and stop codons are in bold. The codons encoding the six histidine residues were included at the 3' end of the antisense primers and are underlined. *Eco*RI and *Kpn*I restriction enzyme sites are shown in lower case and the linker region is in italic. The A/T cloning vector pGEM-T was used in cloning in the current study, so the restriction enzyme sites were not used.

Primer Name BM#	Descriptive Primer Name	Primer Sequence
BM91	11639F	5' *ATA T*ga att c***TA AGG AGG TCT AAA*** **ATG** TTC CAA CCC CTA TTA GAC GCT 3'
BM101	UA948F	5' *ATA T*ga att c***TA AGG AGG TCT AAA*** **ATG** TTC CAG CCC TTA CTA GAC GCT 3'
-	$11639^{1\text{-}447}$R	5' *CGC* gga tcc **TTA** GTG ATG GTG ATG GTG ATG GGA TAA TTC CAA AAG AGG GGT AGC 3'
-	$UA948^{1\text{-}434}$R	5' *CGC* gga tcc **CTA** GTG ATG GTG ATG GTG ATG GGA TAG CTC CAA TAA AGG GGT AGC 3'
-	$11639^{1\text{-}441}$R	5' *CGC* gga tcc **TTA** GTG ATG GTG ATG GTG ATG GGT AGC TTT TGA TAA GAG GCG TTC 3'
-	$UA948^{1\text{-}428}$R	5' *CGC* gga tcc **CTA** GTG ATG GTG ATG GTG ATG GGT AGC CTT TGA TAA GAG GCG TTC 3'
-	$11639^{1\text{-}363}$R	5' CGC gga tcc TTA GTG ATG GTG ATG GTG ATG AAT AGT TAC TAA AGG CTC 3'
-	$UA948^{1\text{-}364}$R	5' CGC gga tcc CTA GTG ATG GTG ATG GTG ATG AAC AGA CAC TAA CGG CTC 3'
BM118	$11639^{1\text{-}370}$	5' **TTA** GTG ATG GTG ATG GTG ATG ATA ATT AAC CCT CAA ATC ATC AAT AGT TAC TAA AGG CTC ATT C 3'
BM128	$UA948^{1\text{-}371}$	5' **CTA** GTG ATG GTG ATG GTG ATG ATG ATC TCT TCT CAA ATC ATC AAC AGA CAC TAA CGG CTC ATT C 3'
BM11	11639, UA948F	5' GCG ATA CCT TTT GCT CTT GTG 3'
BM12	11639, UA948R	5' TGG AGT TTT GAA GTG GTG GAT G 3'
BM98	$11639^{1\text{-}HepRep}$R	5' ATA ATT AAC CCT CAA ATC ATC AAT AGT TAC TAA AGG CTC ATT C 3'
BM99	$11639^{1\text{-}HepRep}$F	5' GAT GAT TTG AGG GTT AAT TAT GAG CGC CTC TTA TCA AAA GCT ACC 3'
BM108	$UA948^{1\text{-}HepRep}$R	5' ATG ATC TCT TCT CAA ATC ATC AAC AGA CAC TAA CGG CTC ATT C 3'
BM109	$UA948^{1\text{-}HepRep}$F	5' GAT GAT TTG AGA AGA GAT CAT GAA CGC CTC TTA TCA AAG GCT ACC 3'
BM96	$11639^{0\text{-}HepRep}$R	5' GGT AGC TTT TGA TAA GAG GCG CTC AAT AGT TAC TAA AGG CTC ATT C 3'
BM97	$11639^{0\text{-}HepRep}$F	5' G AAT GAG CCT TTA GTA ACT ATT GAG CGC CTC TTA TCA AAA GCT ACC 3'
BM77	11639R	5' *TAT A*gg tac c**TT** AGT GAT GGT GAT GGT GAT G*TG C*CA AAC CCA ATT TTT TAA CCC A 3'
BM87	UA948R	5' *TAT A*gg tac c**CT** AGT GAT GGT GAT GGT GAT G*TG C*TT TTC TAA CCC ACC TCC 3'
BM79	$11639^{10\text{-}447}$F	5' *ATA* Tga att c***TA AGG AGG TCT AAA*** **ATG** AGC GCT TCC ATT GAA AAA ATG 3'
BM89	$UA948^{10\text{-}434}$F	5' *ATA* Tga att c***TA AGG AGG TCT AAA*** **ATG** AGC ACC CAT TTA GAT GAA ACA 3'

A 500 ml volume of cells was harvested, resuspended in HEPES buffer (20 mM, pH 7.0 containing 0.5 mM PMSF) and lysed by three passages through a French Press (American Instrument Company, Silver Springs, MD) at 12,000 psi. Cell debris was removed by centrifugation at 8,000 × g for 10 min and the supernatant was subsequently centrifuged at 40,000 × g for 2 h to separate the soluble fraction (cytosol and perplasm) and the membrane fraction (pellet).

4.2.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Immuno-blot Analysis

Cell crude extracts, soluble or membrane fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The total protein concentration was determined by the BCA assay using BSA as protein standard. FucT expression was detected by Western immuno-blotting with mouse anti-penta-histidine monoclonal antibody (1:1000). The density of the FucT band was quantified and used to normalize the FucT activity as described previously (Ma *et al.*, 2003).

4.2.6 Radiochemical Assays of ΔFucTs

Enzyme activities of each truncated FucT in crude cell extracts was assayed with donor GDP-Fuc at 200 μM, GDP-^{3}[H]Fuc at 0.2 μM (~60,000 dpm) and Type II-R or Type I-R acceptors at 1.8 mM or 7.5 mM, respectively (Ge *et al.*, 1997; Ma *et al.*, 2003; Rasko *et al.*, 2000b). The reverse phase C_{18} cartridges were used to isolate the products (containing the hydrophobic aglycone and the acquired radiolabeled fucose moiety) from the un-reacted GDP-^{3}[H]Fuc as described previously (Palcic, 1988). One milliunit (mU) represents the amount of enzyme that converts 1 nmol of acceptor substrate to product per min. The specific activity (mU mg^{-1}) was obtained by dividing the enzyme activity (mU) by the amount of total protein. The activity below 0.01 mU mg^{-1} was considered undetectable. The specific enzyme activity of each FucT was subsequently standardized by FucT expression level, which was determined by immuno-blot.

4.2.7 Purification of 11639$^{1-441}$ and UA948$^{1-428}$

The cell lysates from 1L of HSMDE3 11639$^{1-441}$ and UA948$^{1-428}$culture were prepared as described above except the harvested cells were resuspended in loading buffer (20 mM HEPES, pH 7.5, 50 mM imadazole, 300 mM NaCl, 0.5 mM PMSF). Following French press, the cell debris was removed by centrifugation at 10,000 × g for 20 min. The supernatant was retained as a crude cell extract, and was subsequently passed through a 0.45 μm filter to remove any remaining particles. The filtered cell extract was loaded onto a HisTrap chelating HP column that had been equilibrated with column buffer (20 mM HEPES, pH 8.0, 50 mM NaCl) on an AKTA Purifier (GE Healthcare, Amersham Biosciences) at 4°C. The column was washed with column buffer and the FucT protein was eluted with 20X column volume of elution buffer (20 mM HEPES pH 8.0, 50 mM NaCl) containing a gradient of imidazole (0.05-1 M) at a flow rate of 1 ml min^{-1}. The fraction with FucT activity were pooled and loaded onto a Source 30Q anion exchange column, which had been equilibrated with 20 mM HEPES (pH 8.0). The anion exchange column was washed with 20 mM HEPES (pH 8.0) and eluted with a linear gradient of 0-1 M NaCl (20X column volume) at a flow rate of 1 ml min^{-1}. As the presence of imidazole in the eluted fractions interferes with the color formation in the BCA assay, protein concentration was determined using a BioRad protein assay kit with BSA as protein standard. The identity of the purified FucTs was assessed by SDS-PAGE stained with Coomassie blue and the purity was quantified by UN-SCAN-IT gel software (Silk Scientific Corporation, Orem, UT).

4.2.8 Storage Conditions of Purified FucTs

To determine the best conditions to store purified enzyme (11639FucT$^{1-441}$ and UA948FucT$^{1-428}$), the purified enzyme preparations were stored at 4°C, -20°C, and -80°C, with or without the addition of 20% or 50% glycerol. In addition, a 100 μl aliquot of purified enzyme was lyophilized and kept at -80°C. At two-week, one-month, two-month and three-month time points, the specific enzyme activities using Type II-R (1.8 mM) from the ten different storage conditions were determined as described above. Taking the α1,3 activity at day one as 100%, the relative levels (%) of enzyme specific activity from the ten different conditions at the various time points was obtained.

4.2.9 Single Substrate Kinetics of WT and 11639$^{1-441}$ and UA948$^{1-428}$ in Cell Crude Extracts

The acceptor and donor kinetics for the full-length and 11639FucT$^{1-441}$ and UA948FucT$^{1-428}$ in cell crude extracts were carried out as previously described (Ma *et al.*, 2003; Ma *et al.*, 2005). Kinetic parameters were obtained by fitting the initial rate data to the Michaelis-Menten equation using non-linear regression analysis with Prism 4.0 software (GraphPad, San Diego, CA).

4.2.10 Dual Substrate Kinetics with Purified 11639FucT$^{1-441}$ and UA948$^{1-428}$

Steady-state dual substrate kinetics was carried out on purified 11639FucT$^{1-441}$ and UA948FucT$^{1-428}$ enzyme preparations. Seven different concentrations of the donor and acceptor were used and GDP-^{3}[H]Fuc (0.8 μM) was included in each reaction. The amount of substrate consumed was less than 15% to ensure linear initial reaction rates.

Data was analyzed as described previously (Marcus *et al.*, 2003) by fitting to the rate equation

$$v = \frac{V_{\max}[A][B]}{(K_{ia}K_b + K_a[B] + K_b[A] + [A][B])}$$

where $[A]$ and $[B]$ represent the concentration of acceptor and donor, respectively. K_a is the Michaelis constant for acceptor, K_b is the Michaelis constant for donor, and K_{ia} is the dissociation constant for acceptor.

4.2.11 Acceptor Hydroxyl Group Mapping with $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$

Purified $11639FucT^{1\text{-}441}$ and $UA948^{1\text{-}428}$ enzyme preparations were used to map the key polar groups of acceptors essential for enzyme recognition. The enzyme activities using Type II-R **(1)**, Type I-R **(13)**, Type II series- **(2)-(12)**, and Type I series- **(14)-(18)** modified acceptors were determined with donor GDP-Fuc at 200 μM, GDP-^{3}[H]Fuc at 0.2 μM (~60,000 dpm) and each acceptor at 2 mM. Taking the enzyme specific activity Type II-R **(1)** or Type I-R **(13)** as 100%, the relative enzyme activity using the Type II series- or Type I series- modified acceptors were obtained and expressed as a percentage, respectively.

4.2.12 Synthesis of Lewis X or Lewis A using Purified $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$

Type II-R (2 mg, 3.6 μmol) and GDP-Fuc (3.5 mg, 5.4 μmol) were used for Lewis X synthesis with purified $11639FucT^{1\text{-}441}$ in 1.5 ml HEPES (20 mM, pH 7.0) buffer containing 100 mM NaCl, 35 mM $MgCl_2$, 5 mg ml^{-1} BSA, 20 mM $MnCl_2$. The reaction was started by the addition of 180 mU of purified $11639FucT^{1\text{-}441}$ and 2 U of alkaline phosphatase. The reaction was gently mixed on an end-over-end shaker at room temperature for 20 h. It has been shown that GDP and GMP are strong inhibitors of human FucT V with K_is of 0.03 mM and 0.70 mM respectively while guanosine has a K_i>10 mM (Qiao *et al.*, 1996). Alkaline phosphatase was therefore added to degrade GDP to GMP and guanosine to minimize the inhibitory effects of the former two compounds. In the same manner, purified UA948FucT$^{1\text{-}428}$ was incubated with 5.2 mg (9.4 μmol) of Type II-R and 8.6 mg (13.5 μmol) of GDP-Fuc; or 3.2 mg (6 μmol) of Type I-O-R and 5.7 mg (9 μmol) GDP-Fuc for Lewis X or Lewis A synthesis, respectively. The Lewis X and Lewis A products were characterized by thin layer chromatography (TLC) and ^{1}H-NMR spectroscopy analysis.

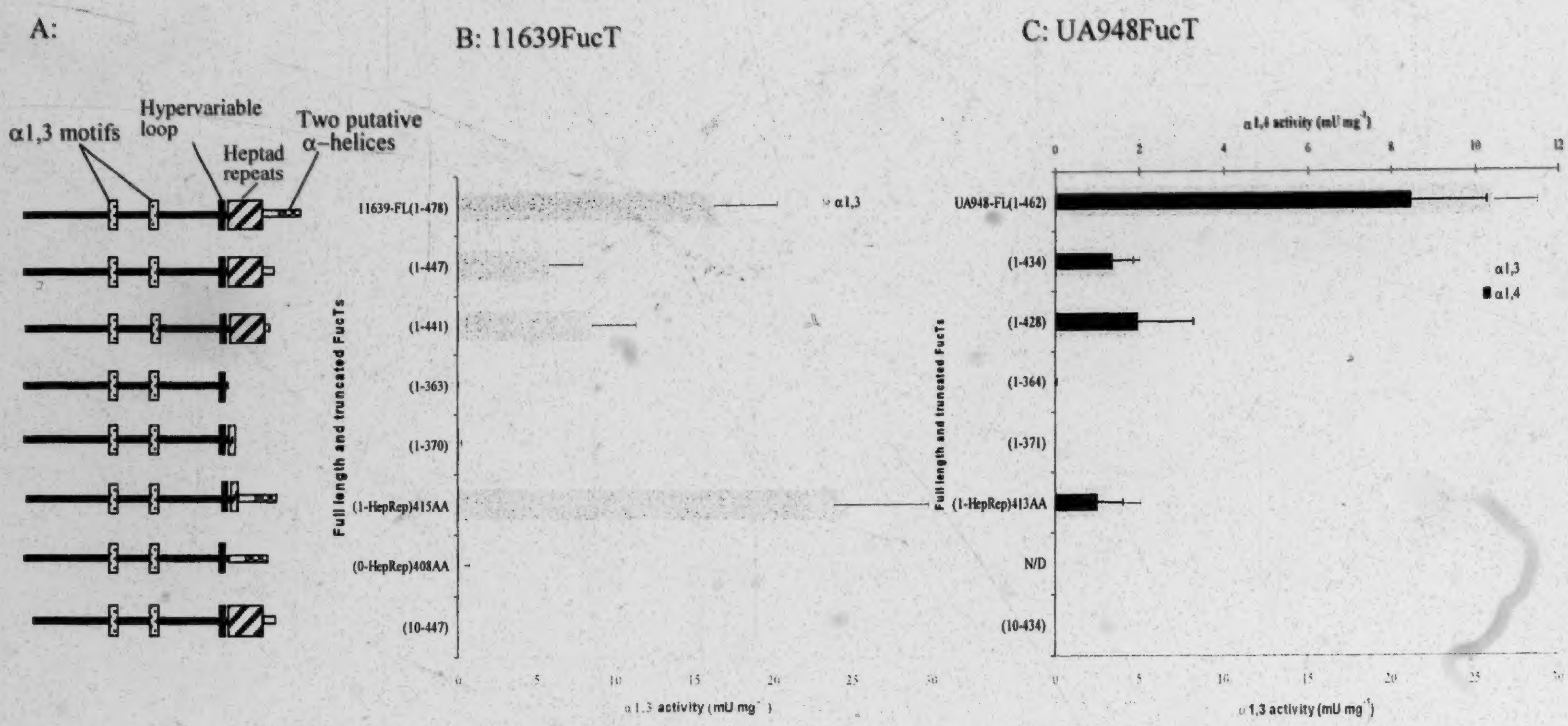
4.3 Results

4.3.1 Mapping of the Minimal Catalytic Domain

In an effort to aid FucT overexpression, purification and future structural determinations, the minimal catalytic domain of *H. pylori* FucTs was mapped by truncating either the N-terminus or the C-terminus of 11639FucT and UA948FucT. A schematic diagram depicting the full-length (478 amino acids for 11639FucT and 462 amino acids for UA948FucT) and the ΔFucTs is shown in Fig. 4-1A. 11639FucT is primarily an α1,3 FucT with very low level of α1,4 activity (Ma *et al.*, 2005), thus only the α1,3 activity of 11639FucT and its truncated constructs were determined. In contrast, both the α1,3 and α1,4 activities of UA948FucT and its truncated mutants were determined by radiochemical assays. Enzyme activity of WT and each ΔFucT was standardized by FucT protein expression level, which was detected by immuno-blotting.

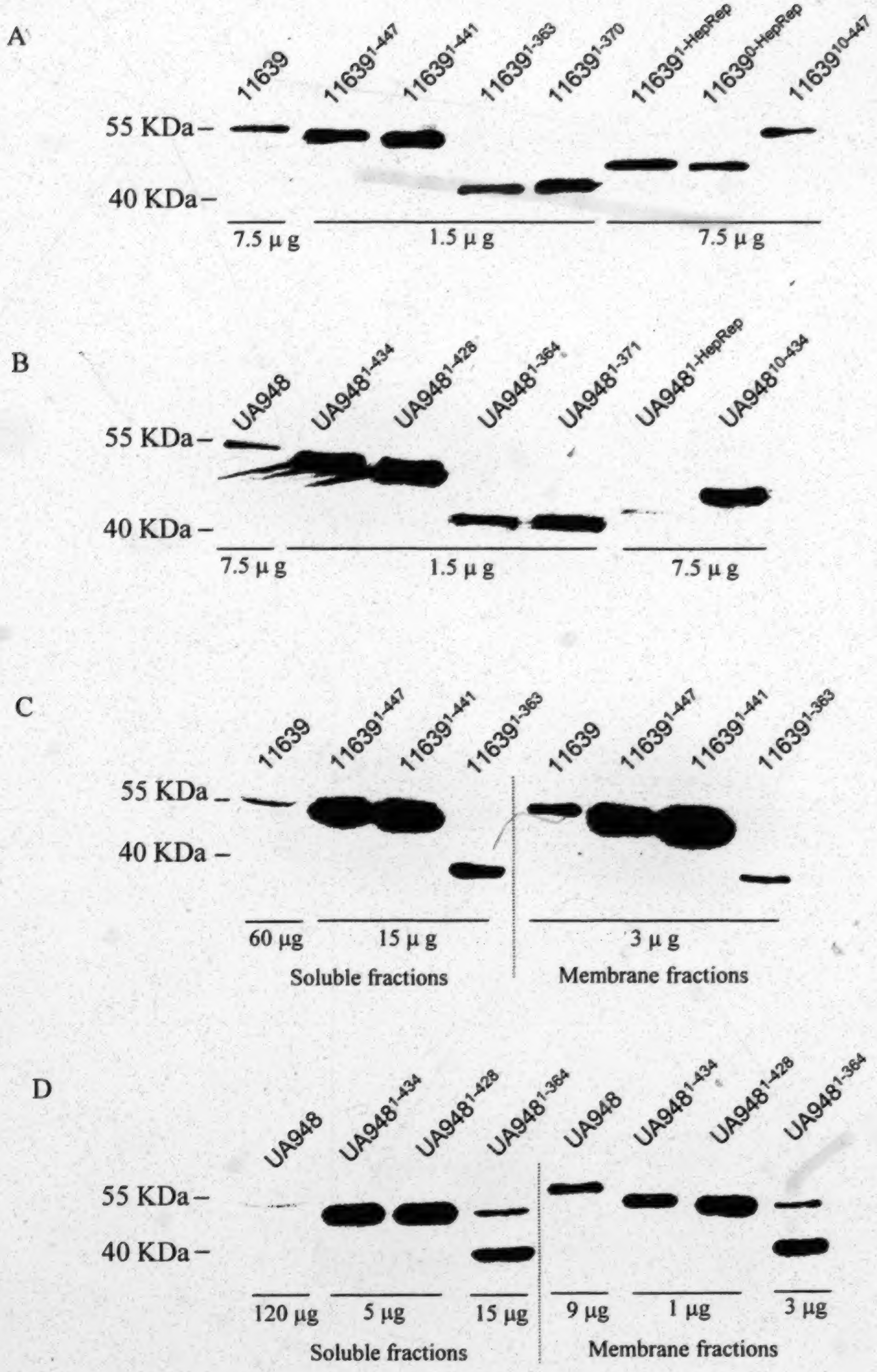
To establish if the two putative amphipathic α-helices at the C-terminus were essential for enzyme activity, 11639FucT$^{1\text{-}447}$, 11639FucT$^{1\text{-}441}$, UA948$^{1\text{-}434}$ and UA948FucT$^{1\text{-}428}$ were constructed by removing those α-helices (Fig. 4-1A). In WT full-

Fig. 4-1. Schematic representation of the truncated FucTs derived from 11639FucT and UA948FucT and their α1,3 and α1,4 activities. A: Schematic representation of the full length and ΔFucTs. The black bar represents the domain of FucT preceding the heptad repeat region. The heptad repeat region is shown as a hatched vertical block. The two putative C-terminal amphipathic helices are shown as a checked bar. The region connecting the heptad repeats and the amphipathic helices is shown as a small white bar. Two α1,3 FucT motifs are shown as narrow dotted vertical blocks. The hypervariable loop that confers Type I acceptor specificity is shown in a narrow black vertical block. **B,C:** The specific enzymatic activities of the full-length (FL) and truncated FucTs derived from 11639FucT (B) and UA948FucT (C), respectively, were standardized by FucT expression levels, which were detected by immuno-blotting. The α1,3 activity is shown in gray bars and the α1,4 activity is shown in black bars. Each value represents the average of three independent determinations with standard deviation indicated. 11639FucT is primarily an α1,3 FucT with very low amount of α1,4 activity (44) thus α1,4 activity was not determined.


A:
α1,3 motifs
Hypervariable loop
Heptad repeats
Two putative α–helices
B: 11639FucT
11639-FL(1-478)
(1-447)
(1-441)
(1-363)
(1-370)
(1-HepRep)415AA
(0-HepRep)408AA
(10-447)
Full length and truncated FucTs
α1,3
0
5
10
15
20
25
30
α1,3 activity (mU mg⁻¹)
C: UA948FucT
α1,4 activity (mU mg⁻¹)
0
2
4
6
8
10
12
UA948-FL(1-462)
(1-434)
(1-428)
(1-364)
(1-371)
(1-HepRep)413AA
N/D
(10-434)
Full length and truncated FucTs
α1,3
α1,4
0
5
10
15
20
25
30
α1,3 activity (mU mg⁻¹)

length 11639FucT and UA948FucT, 19 amino acids are present connecting the heptad repeat region with the C-terminal putative amphipathic helices (Fig. 4-1A, shown as a small white bar). In 11639FucT$^{1\text{-}447}$, UA948$^{1\text{-}434}$, 11639FucT$^{1\text{-}441}$ and UA948FucT$^{1\text{-}428}$, 14 or 8 amino acids remain in the connecting region (Fig.4- 1A, shown as a small white bar) in the former and latter pair of constructs, respectively. The enzyme activities of these four ΔFucTs were about 3-8 times higher than those of the full-length proteins before considering how much FucT was expressed. As shown in Fig.4-2A, 4-2B, the expression levels of these four ΔFucTs in cell crude extract were much higher than those of the full-length proteins (about 10-37 times). After standardization of the activities with FucT expression levels, the specific enzyme activity of 11639FucT$^{1\text{-}447}$, 11639FucT$^{1\text{-}441}$, UA948$^{1\text{-}434}$ and UA948FucT$^{1\text{-}428}$ turned out to be lower than that of the full-length proteins (Fig. 4-1B, 4-1C). The improved protein expression was reflected in the augmentation of protein content in both soluble and membrane fractions (Fig. 4-2C, 4-2D). Meanwhile the total enzyme activity was also increased in both the soluble and membrane fractions after removal of the putative α-helices, however the degree of the increase in soluble fraction was 2-3 fold of that in the membrane fraction, indicating that a portion of the enzyme activity was shifted to the soluble fraction. Nevertheless, a significant amount of protein was still observed in the membrane fractions when the amphipathic helices were deleted (11639FucT$^{1\text{-}447}$, 11639FucT$^{1\text{-}441}$, UA948$^{1\text{-}434}$ and UA948FucT$^{1\text{-}428}$) (Fig. 4-2C, 4-2D), suggesting that the C-terminal amphipathic α-helices, which were proposed to function as a membrane anchor (Ma *et al.*, 2003), are neither required for enzyme activity nor the sole determinants for membrane association.

Fig. 4-2. Immuno-blot of full length and truncated FucTs. The full length and truncated FucTs with His_6 tag were expressed in *E. coli* HMS174DE3 cells with induction using 1 mM IPTG and growth at 30°C for 4~5 h. The FucT protein was detected with mouse anti-penta-histidine monoclonal antibody. The amount of total protein loaded is shown on the bottom of each lane. **C** and **D:** Soluble and membrane fractions are also labeled.


A
11639
11639^{1-447}
11639^{1-441}
11639^{1-363}
11639^{1-370}
$11639^{1-HepRep}$
$11639^{0-HepRep}$
11639^{10-447}
55 KDa
40 KDa
7.5 µ g
1.5 µ g
7.5 µ g
B
UA948
$UA948^{1-434}$
$UA948^{1-428}$
$UA948^{1-364}$
$UA948^{1-371}$
$UA948^{1-HepRep}$
$UA948^{10-434}$
55 KDa
40 KDa
7.5 µ g
1.5 µ g
7.5 µ g
C
11639
11639^{1-447}
11639^{1-441}
11639^{1-363}
11639
11639^{1-447}
11639^{1-441}
11639^{1-363}
55 KDa
40 KDa
60 µg
15 µ g
3 µ g
Soluble fractions
Membrane fractions
D
UA948
$UA948^{1-434}$
$UA948^{1-428}$
$UA948^{1-364}$
UA948
$UA948^{1-434}$
$UA948^{1-428}$
$UA948^{1-364}$
55 KDa
40 KDa
120 µg
5 µg
15 µg
9 µg
1 µg
3 µg
Soluble fractions
Membrane fractions

To determine whether the entire heptad repeat region was required for FucT activity $11639^{1\text{-}363}$, $\text{UA948}^{1\text{-}364}$ and $11639^{0\text{-HepRep}}$ were constructed. After removal of the entire heptad repeat region, these three ΔFucTs exhibited an extremely low level of enzyme activity, although protein expression was either moderately higher than ($11639^{1\text{-}363}$, $\text{UA948}^{1\text{-}364}$) or similar to ($11639^{0\text{-HepRep}}$) those of full-length FucTs (Fig. 4-2). This indicated that the heptad repeat region is essential for enzyme activity.

To determine if a shorter heptad repeat region was sufficient for conferring enzyme activity, constructs $11639^{1\text{-}370}$ and $\text{UA948}^{1\text{-}371}$ were made, which are truncated immediately after the first heptad repeat. In addition, $11639^{1\text{-HepRep}}$ and $\text{UA948}^{1\text{-HepRep}}$ that contain one heptad repeat plus the downstream 19 amino acids linker and the two putative amphipathic α-helices were also constructed (Fig. 4-1A). Constructs $11639^{1\text{-}370}$ and $\text{UA948}^{1\text{-}371}$ had little enzyme activity (Fig. 4-1B, 4-1C). Remarkably, $11639^{1\text{-HepRep}}$ displayed enzyme activity comparable to that of the full-length protein. This suggests that a much shorter "stem" region, and precisely one heptad repeat, is sufficient for full activity of 11639FucT (Fig. 4-1B). In contrast, UA948FucT with one heptad repeat ($\text{UA948}^{1\text{-HepRep}}$) exhibited partial activity compared to that of the full-length enzyme (Fig. 4-1C). It was consistently observed that $\text{UA948}^{1\text{-HepRep}}$ displayed a lower protein expression level than the full-length protein and the other constructs (Fig. 4-2B), suggesting that this construct was either poorly expressed or the expression was unstable. Although $\text{UA948}^{0\text{-HepRep}}$ was constructed, it was not possible to characterize this mutant due to the lack of protein expression. It is possible that deletion of the entire heptad repeat region of UA948FucT causes folding or degradation problems that prevent proper protein expression.

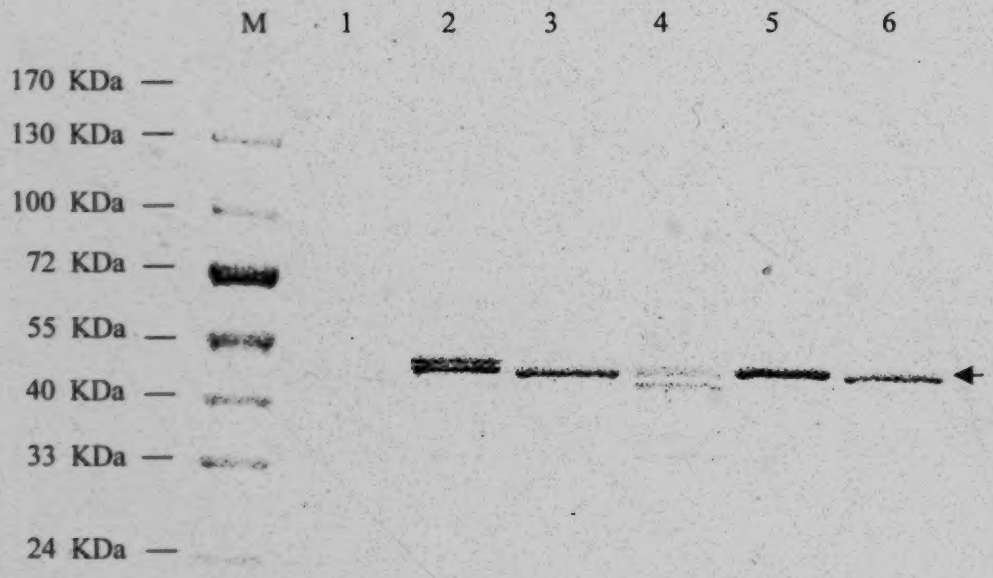
Lastly, to determine whether or not the N-terminus of *H. pylori* FucTs is essential for function, 11639$^{10-447}$ and UA948$^{10-434}$ were constructed by deleting ten amino acids at the N-terminus of 11639$^{1-447}$ and UA948$^{1-434}$, respectively. These two truncated constructs completely lost enzyme activity (Fig. 4-1B, 4-1C), despite the fact that both were expressed at a similar level as full-length enzymes (Fig. 4-2A, 4-2B). This demonstrates the necessity of the N-terminal residues for enzyme function.

4.3.2 Purification of 11639$^{1-441}$ and UA948$^{1-428}$

Removal of the C-terminal helices greatly improved protein expression and solubility while maintaining enzyme activity at a significant level (Fig. 4-1 and Fig. 4-2). Therefore 11639FucT$^{1-441}$ and UA948FucT$^{1-428}$ with a His6-tag at the C-terminus were purified by a sequence of immobilized metal affinity chromatography (IMAC) and anion exchange chromatography (AIEX). The calculated molecular weights of 11639FucT$^{1-441}$ and UA948FucT$^{1-428}$ are 52.5 kDa and 51.2 kDa respectively. Prior to purification, the FucT bands were visible at the predicted position but did not stand out on a Coomassie-blue stained SDS-PAGE gel (Lanes 1 and 4 in Fig. 4-3). The two-step purification resulted in 11639$^{1-441}$ and UA948$^{1-428}$ enzyme with 78.2% and 79.7% purity, respectively, quatified by UN-SCAN-IT (Fig. 4-3).

From 1 L cultures, 6.7 mg and 4.4 mg of FucT protein was isolated, and the enzyme specific activity was increased from 0.05 U mg^{-1} to 3.7 U mg^{-1} and 2.8 U mg^{-1} by 77- and 52-fold for 11639FucT$^{1-441}$ and UA948FucT$^{1-428}$, respectively. It is worth noting that UA948$^{1-428}$ was rather unstable after the IMAC and would precipitate if the fractions were left at 4°C for 30-60 min. Therefore, the FucT fractions eluted from the IMAC column were loaded immediately onto the AIEX column. Even so, a significant drop in

Fig. 4-3. SDS-PAGE electrophoresis of FucT proteins before and after purification. The gel was stained with Coomassie blue. Total protein of 6 μg (crude extract, Lane 1, 4) or 3 μg (purified fractions, Lane 2, 3, 5, 6) was loaded. Lane 1-3, 11639FucT$^{1\text{-}441}$; Lane 4-6, UA948FucT$^{1\text{-}428}$. Lane 1,4, cell crude extract; Lane 2,5, pooled IMAC purification fractions; Lane 3,6, pooled AIEX purification fractions. The arrow indicates the positions of 11639$^{1\text{-}441}$ and UA948$^{1\text{-}428}$ on the blot.


M
1
2
3
4
5
6
170 KDa
130 KDa
100 KDa
72 KDa
55 KDa
40 KDa
33 KDa
24 KDa

both total and specific enzyme activity was observed during the AIEX purification procedure.

4.3.2 Storage and Stability

To determine the best storage method for the purified FucTs, the α1,3 activity was monitored by radiochemical assays following enzyme storage after two-week, one-month, two-month or three-month at three different temperatures (4°C, -20°C and -80°C) with different concentrations of glycerol (0%, 20% or 50%). In addition, one sample was lyophilized and stored at -80°C. Lyophilized $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$ lost 38% and 23% activity after three months, respectively. Addition of glycerol (20% or 50%) significantly stabilized the enzyme preparations at both 4°C and -20°C, with little loss of activity after three months. FucT preparations were most stable at -80°C with or without glycerol supplementation, thus this condition was adopted for the routine storage protocol.

4.3.4 Single Substrate Kinetics of WT and $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$ in Crude Extracts

Removal of the C-terminal putative amphipathic helices facilitated the overexpression and purification of *H. pylori* α1,3/4 FucTs, however it was not known whether or not the truncation affected the kinetic parameters for the enzyme. The single substrate kinetics of WT and constructs $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$ in cell crude extracts were characterized and compared.

The acceptor kinetic data showed that truncation of the C terminus did not cause any modification of the Type II acceptor kinetics for 11639FucT, however although the acceptor K_m remained unchanged for UA948FucT, the V_{max} decreased by 50% (Table 4-2). With Type I acceptor, $UA948^{1\text{-}428}$ possessed a 2-fold tighter acceptor binding affinity,

Table 4-2. The acceptor and donor kinetic parameters of wild type and truncated FucTs. Acceptor kinetics were determined in cell crude extracts using 0.03-2 mM Type II-R, or 0.4-25 mM Type I-R with GDP-Fuc at 200 μM and GDP-^{3}[H]Fuc at 0.2μM. Donor kinetics were determined using 3-200 μM GDP-Fuc with Type II-R at 2 mM or Type I-R at 15 mM and GDP-^{3}[H]Fuc at 0.8 μM.

Full-length &	Type II-R[b]			Type I-R[c]			K_m for GDP-Fuc (μM)	
Truncated[a]	K_m (mM)	V_{max}[d]	V_{max} / K_m[e]	K_m (mM)	V_{max}	V_{max} / K_m[e]	Type II-R[b]	Type I-R[c]
11639FucT	0.37 ± 0.03	11.8 ± 0.3	31.9	–[f]	–[f]	–[f]	44.7 ± 5.5	–[f]
UA948FucT	1.5 ± 0.2	29.0 ± 1.8	19.3	13.4 ± 1.4	11.7 ± 0.6	0.87	56.0 ± 10.1	33.7 ± 7.8
11639$^{1\text{-}441}$	0.39 ± 0.07	12.0 ± 0.8	30.8	–[f]	–[f]	–[f]	15.6 ± 2.4	–[f]
UA948$^{1\text{-}428}$	1.7 ± 0.2	16.1 ± 1.0	9.5	5.7 ± 0.5	5.0 ± 0.3	0.88	17.6 ± 3.7	11.9 ± 0.8

[a] All FucTs possessed a His$_6$-tag at the C-terminus.

[b] Type II-R: Galβ1,4GlcNAc-O$(CH_2)_8CO_2CH_3$.

[c] Type I-R: Galβ1,3GlcNAc-O$(CH_2)_8CO_2CH_3$.

[e] V_{max} / K_m : mU mg^{-1} mM^{-1}.

[d] V_{max} (mU mg^{-1}) was standardized based on FucT expression quantified by immuno-blot.

-[f]: not determined due to the very low level of α1,4 activity thus the kinetic parameters could not be obtained with confidence.

which exactly compensated for the 2-fold reduction of the V_{max} value when calculating the catalytic efficiency (V_{max}/K_m) of the enzyme. Notably, the C-terminal truncation improved the donor binding by about 3-fold with both Type II and Type I acceptors for both FucTs.

4.3.5 Dual Substrate Kinetics of Purified 11639^{1-441} and $UA948^{1-428}$

The success in purifying milligrams per liter of 11639^{1-441} and $UA948^{1-428}$ FucTs allowed us to perform the dual substrate kinetics with seven different concentrations of the donor GDP-Fuc and seven different concentrations of acceptor (Type II or Type I). GDP-^{3}[H]Fuc at 0.8 μM was included in each reaction. The kinetic parameters are shown in Table 4-3. The acceptor and donor K_m values for fucose transfer to Type II acceptor by $11639FucT^{1-441}$ and $UA948FucT^{1-428}$ are similar, but those of $11639FucT^{1-441}$ are slightly lower (Table 4-3). The k_{cat} of $11639FucT^{1-441}$ and $UA948FucT^{1-428}$ are 2.3 s^{-1} and 1.1 s^{-1}, respectively, which gives rise to the catalytic efficiency parameters (k_{cat}/K_a and k_{cat}/K_b) of $11639FucT^{1-441}$ with Type II acceptor being approximately three-fold of those of $UA948FucT^{1-428}$.

UA948FucT possessed the same k_{cat} values (1.1 s^{-1}) for Type II and Type I acceptors. However, the much lower K_m for Type II acceptor resulted in a 28-fold k_{cat}/K_a with Type II of that with Type I acceptor, therefore UA948FucT strongly favors Type II over Type I acceptor. For the donor GDP-Fuc, $UA948FucT^{1-428}$ displayed a slightly tighter binding when fucose is transferred to Type I versus to Type II acceptor. The acceptor and donor K_m values obtained from dual substrate kinetics with the purified enzyme (Table

Table 4-3. Dual substrate kinetic parameters of the purified truncated 11639FucT$^{1-441}$ and UA948FucT$^{1-428}$ with donor (GDP-Fuc) and acceptor (Type II or Type I). k_{cat} is the catalytic turnover rate, K_a is the Michaelis constant for acceptor, K_b is the Michaelis constant for donor, K_{ia} is the dissociation constant for acceptor, K_{ib} is the dissociation constant for donor. The α values reflect the effect of binding one substrate on the binding of the other.

Enzyme[a]	11639FucT$^{1-441}$[b]	UA948FucT$^{1-428}$	
Acceptor	Type II	Type II	Type I
k_{cat} (s^{-1})	2.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
K_a (μM/mM)	305 ± 32 μM	397 ± 79 μM	8.3 ± 0.7 mM
K_b (μM)	10.4 ± 1.5	14.4 ± 3.7	8.1 ± 1.7
K_{ia} (μM/mM)	95 ± 16 μM	127 ± 6 μM	2.2 ± 0.4 mM
K_{ib} (μM)	3.2 ± 1.2	5.3 ± 0.9	1.6 ± 0.4
α	3.3 ± 0.1	2.9 ± 0.2	4.4 ± 0.6
k_{cat}/K_a ($\times 10^3$ s^{-1} M^{-1})	7.5	2.8	0.1
k_{cat}/K_b ($\times 10^3$ s^{-1} M^{-1})	221	76	136
$k_{cat}/K_{ia}K_b$ ($\times 10^9$ s^{-1} M^{-1})	2.3	0.6	0.06

[a]: Both FucT enzymes have a His$_6$-tag at the C-terminus.

[b]: 11639FucT is primarily an α1,3 FucT with trace amount of α1,4 activity (Ma *et al.*, 2005) thus the kinetic parameters of 11639$^{1-441}$ with Type I acceptor were not determined.

4-3) are lower than those obtained from single substrate kinetics with cell crude extracts (Table 4-2), except for the K_m using Type I acceptor for UA948$^{1-428}$.

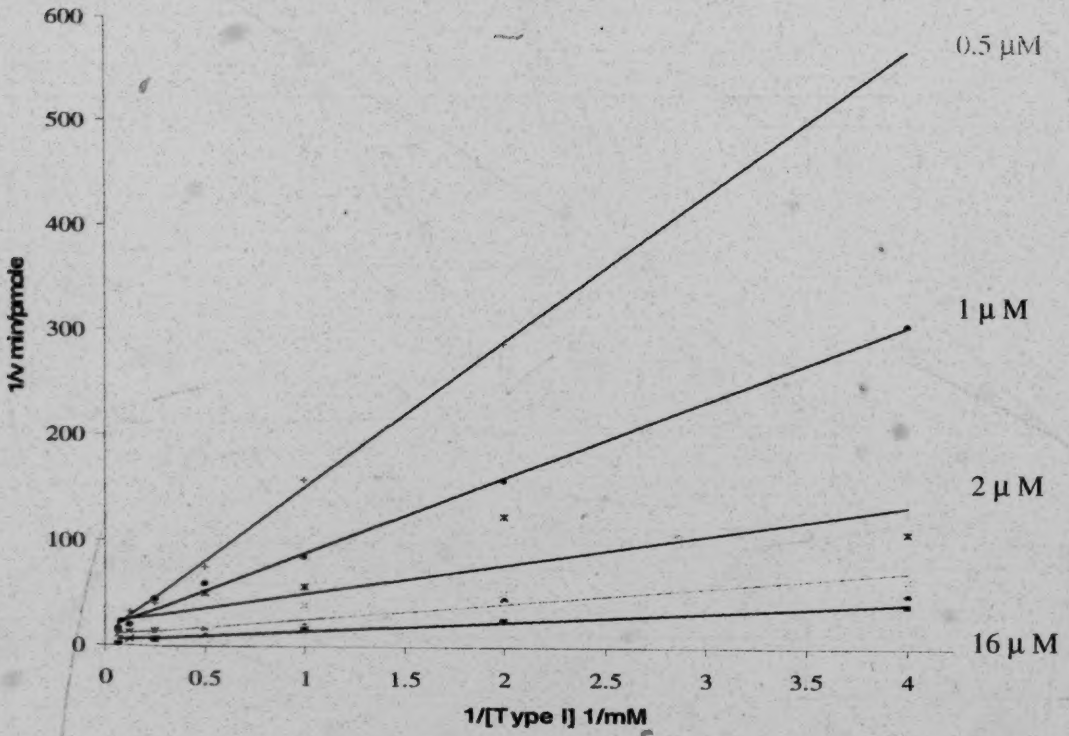
To determine the kinetic mechanism of 11639$^{1-441}$ and UA948$^{1-428}$, a pattern of double reciprocal plots for velocity and concentration of either substrate at a series of fixed concentrations of the second substrate was produced. The resulting family of intersecting lines (Fig. 4-4) excludes a double displacement (ping-pong) mechanism. As such it is concluded that the catalytic mechanism of *H. pylori* FucTs is sequential and both substrates bind to the enzyme before any product is released.

4.3.6 Acceptor Hydroxyl Group Mapping with *H. pylori* FucTs

To map the molecular determinants in Type II and Type I acceptors that are essential for recognition by *H. pylori* α1,3/4 FucTs, sixteen synthetic acceptors with modifications at the hydroxyl and *N*-acetamido groups were employed. Due to the limited availability of the compounds, the enzyme activities were determined using 2 mM of each acceptor. In comparison to the transfer rate with unmodified Type II acceptor **(1)** or Type I acceptor **(13)**, the relative rates (%) of transfer for each modified acceptor were obtained (Table 4-4).

Purified 11639$^{1-441}$ and UA948$^{1-428}$ FucTs exhibited variable levels of enzyme activity using twelve different Type II–series acceptors (Table 4-4). Substitution of the hydroxyl group at C-3 of the galactose with a sulfate group **(2)** and replacement of the hydroxyl group at C-2 of the galactose with fucose **(3)** resulted in enhanced or comparable rates of transfer for 11639$^{1-441}$ and UA948$^{1-428}$ respectively relative to the unmodified acceptor **(1)**. Addition of a bulky sialic acid moiety **(4)** greatly reduced the rate of transfer, particularly for UA948FucT (Table 4-4). Modifications at the C-4 position with

Fig. 4-4 Double-reciprocal plot of the initial velocities of UA948$^{1-428}$ with varying acceptor Type I (mM) and fixed donor GDP-Fuc (μM) (top) and varying donor GDP-Fuc (μM) and fixed Type I acceptor (mM) (bottom).


600
500
400
300
200
100
0
1/v min/pmole
0
0.5
1
1.5
2
2.5
3
3.5
4
1/[Type I] 1/mM
0.5 μM
1 μ M
2 μ M
16 μ M

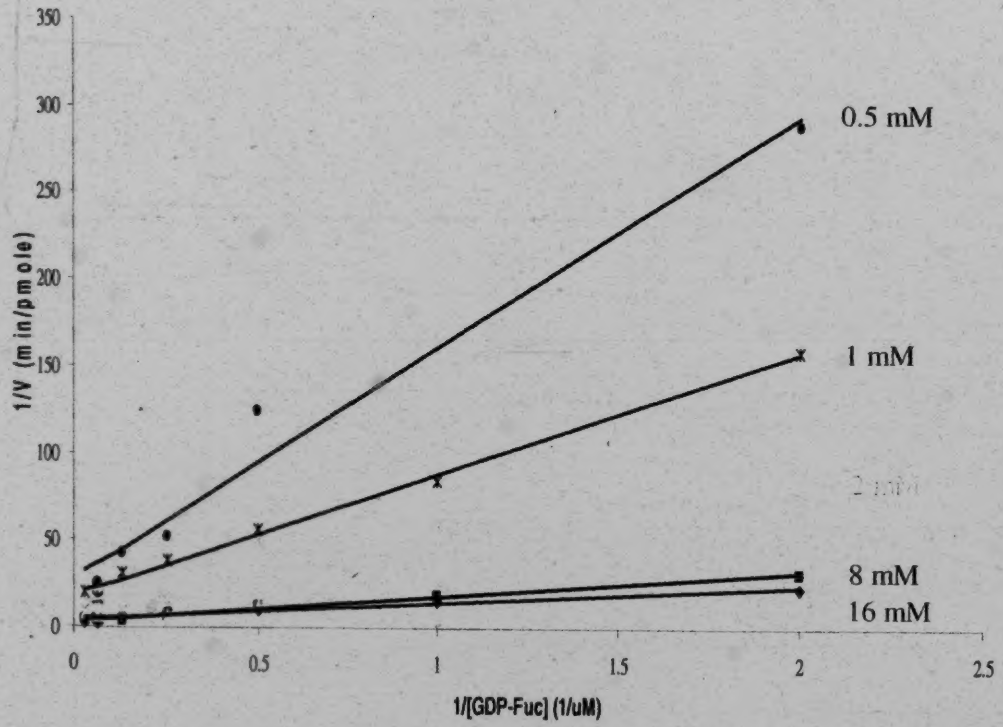

350
300
250
200
150
100
50
0
1/V (min/pmole)
0
0.5
1
1.5
2
2.5
1/[GDP-Fuc] (1/uM)
0.5 mM
1 mM
2 mM
8 mM
16 mM

Table 4-4. Acceptor specificity of the purified recombinant *H. pylori* FucTs for eighteen Type II- and Type I-series oligosaccharides. The relative rates of fucose transfer with Type II-series compounds are expressed as a percentage of incorporation with unmodified Type II acceptor **(1)** for $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$ FucTs, respectively. The relative rates with Type I-series compounds are expressed as a percentage of incorporation with unmodified Type I acceptor **(13)** for purified $UA948^{1\text{-}428}$ FucT. Each acceptor was used at 2 mM. The specific enzyme activities with compound **(1)** for $11639^{1\text{-}441}$ and $UA948FucT^{1\text{-}428}$ are 2.9×10^3 mU mg^{-1} and 8.6×10^3 mU mg^{-1} respectively and with compound **(13)** for $UA948^{1\text{-}428}$ is 1.5×10^3 mU mg^{-1}. These activities correspond to the relative activity of 100%.

Substrate[b]	**Relative activity (%)**	
	$11639^{1\text{-}441a}$	**$UA948^{1\text{-}428a}$**
Type II Series		
(1) Galβ1,4GlcNAc	100	100
(2) 3-Sulfo-Galβ1,4GlcNAc	135	72
(3) Fucα1,2Galβ1,4GlcNAc	115	99
(4) NeuAcα2,3Galβ1,4GlcNAc	32	1
(5) 4-Deoxy-Galβ1,4GlcNAc	28	8
(6) 6-Deoxy-Galβ1,4GlcNAc	0.02	0.02
(7) 6-Sulfo-Galβ1,4GlcNAc	2	0.04
(8) Galβ1,4-6-Phospho-GlcNAc	6	0.4
(9) Galβ1,4Glc	0.4	1
(10) Galα1,3- Galβ1,4Glc	3	2
(11) Galα1,4- Galβ1,4Glc	0.1	0.04
(12) Galβ1,4-3-Deoxy-GlcNAc	-	-
Type I Series		
(13) Galβ1,3GlcNAc	ND	100
(14) 3-Sulfo-Galβ1,3GlcNAc	ND	40
(15) Fucα1,2Galβ1,3GlcNAc	ND	34
(16) NeuAcα2,3-Galβ1,3GlcNAc	ND	0.05
(17) 6-deoxy-Galβ1,3GlcNAc	ND	-
(18) Galβ1,3-4-deoxy-GlcNAc	ND	-

[a]: FucT enzymes have a His_6-tag at C-terminus.

[b]: All Type I- and Type II-series acceptors have an aglycone (β-O-$(CH_2)_8CO_2CH_3$) at C-1 of the GlcNAc moiety.

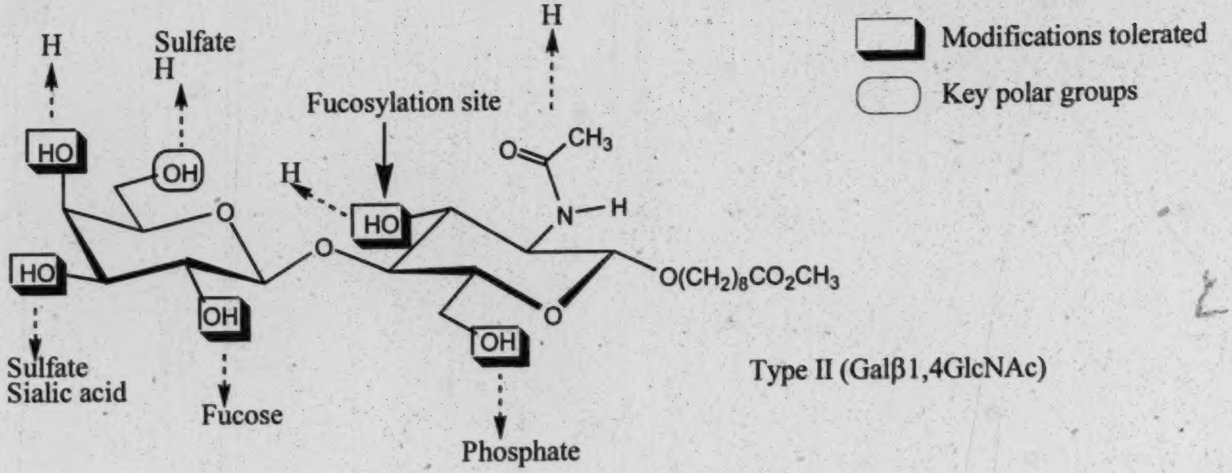
-: Activity lower than 0.01 mU mg^{-1} was considered undetectable.

ND: not determined.

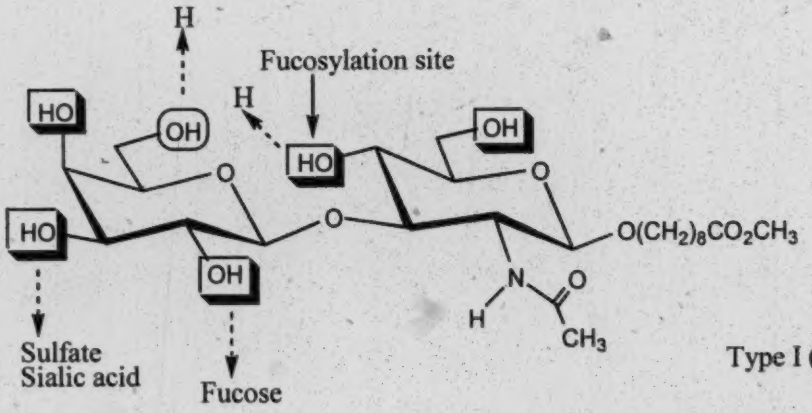
deoxygenation **(5)** reduced the activity about 4-fold and 13-fold for 11639FucT and UA948FucT, respectively (Table 4-4). The most dramatic impact was observed by modifications at the C-6 position. When this hydroxyl was deoxygenated, the compound **(6)** became a very poor substrate for both $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$ with 0.02% of the transfer rate relative to the unmodified Type II acceptor **(1)**. Replacement of the C-6 hydroxyl group by a sulfate group **(7)** gave a slightly improved transfer rate at 2% and 0.04% for $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$ respectively. Such dramatic decrease in activity qualifies the hydroxyl at C-6 of galactose in Type II acceptor as a key polar group required for enzyme recognition (Fig. 4-5). In general, $11639^{1\text{-}441}$ tolerated the modifications on the terminal galactose moiety of Type II acceptor (compounds **(2)** **(4)**, **(5)** and **(6)**) better than $UA948^{1\text{-}428}$. This may be due to the lower K_m for Type II acceptor of 11639FucT than that of UA984FucT (Table 4-2 and Table 4-3).

Modifications of the GlcNAc moiety in Type II acceptor greatly reduced the transfer rate for both enzymes. Phosphorylation of the hydroxyl group at C-6 of the GlcNAc **(8)** caused a large decrease in activity for both $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$. The lack of an N-acetamido group **(9)** also severely impaired enzyme activity, as did the modification of compound **(9)** after adding a galactose at C-3 to generate **(10)** or after adding a galactose at C-4 to create **(11).** Deoxygenation at C-3 of the GlcNAc of Type II substrate **(12)** generated an inactive compound for both enzymes. To evaluate if compound **(12)** could function as an inhibitor, 4.4 mM of compound **(12)** and 55.6 μM of substrate **(1)** were included in the reaction and a 42.5% and 28.3% decrease in activity was observed for $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$, respectively. The limited availability of compound **(12)** precluded from performing a complete kinetic study to obtain an accurate K_i.

Fig 4-5. Structure of the Type II and Type I acceptors and the key polar group mapping for *H. pylori* α1,3/4 FucTs. The broken arrows point to the modifications in our current study.



Type II (Galβ1,4GlcNAc)



Type I (Galβ1,3GlcNAc)

Nevertheless, assuming that the inhibition is competitive, a K_i of 5.0 mM and 9.8 mM could be calculated for $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$ respectively, using the relationship of i=100 [I]/([I] $+K_i$ {1 +[S]/K_m}), where i is the % inhibition, [I] is the inhibitor concentration, [S] is the acceptor concentration, and K_m is the acceptor K_m for compound **(1)**. The high K_i value of compound **(12)** argues for an important role of the OH-3 of Type II acceptor in recognition by 11639FucT and UA948FucT. Such weak inhibition has also been observed for FucTs isolated from human milk when 4-OH of GlcNAc in Type I acceptor was deoxygenated (Du and Hindsgaul, 1996; Gosselin and Palcic, 1996).

Five modified Type I-series analogues were also utilized to map the hydroxyl groups in Type I acceptor that are required for recognition by $UA948^{1\text{-}428}$ (Table 4-4). Substitutions of the hydroxyl group at C-3 of the galactose with a sulfate group **(14)** and the C-2 of the galactose with a fucose group **(15)** were relatively well tolerated. In contrast, the replacement of C-3 of the galactose with sialic acid **(16)** created a very poor acceptor. Notably, deoxygenation at C-6 of the galactose **(17)** rendered the compound completely inactive, indicating that the hydroxyl group at C-6 of the galactose in Type I acceptor is absolutely required for recognition by $UA948^{1\text{-}428}$. Deoxygenation at C-4 of GlcNAc in Type I structure **(18)** made the compound unable to function as an acceptor. When Type I analogue **(18)** at 4.4 mM was evaluated as an inhibitor with **(13)** included in the reaction at 55.6 μM as an acceptor, no inhibition was observed. This indicates that the reactive hydroxyl in Type I acceptor is curcial for enzyme recognition.

4.3.7 Synthesis of Lewis X and Lewis A using Purified $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$

Purified $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$ were used for enzymatic synthesis of Lewis X and Lewis A. The conversion rate from substrate to product in each reaction was monitored by TLC ($CHCl_3$:MeOH:water =70:35:2). When TLC showed the complete disappearance of the substrate and the formation of a single product, the reaction was terminated. The product was isolated by column chromatography on a reverse-phase C_{18} cartridge and subsequently lyophilized, with yields ranging from 87% to 94%. The products (Lewis X and Lewis A) and the starting substrates (Type II and Type I) were analyzed by ^{1}H-NMR spectroscopy (Table 4-5). The chemical shifts for products are identical to literature values for Lewis X and Lewis A (Hounsell *et al.*, 1988; Palcic *et al.*, 1989b).

4.4 Discussion

The presented data demonstrate that the C-terminus, but not the N-terminus, of *H. pylori* α1,3/4 FucTs can be truncated without significant loss in activity. This is in contrast to mammalian α1,3/4 FucTs where truncation of the C-terminus, but not the N-terminus, abolishes activity. This supports the proposed inverted domain model, in which *H. pylori* α1,3/4 FucTs contain the C-terminal heptad repeat region followed by two putative amphipathic α-helices that are functionally equivalent to the N-terminal stem and transmembrane regions respectively in mammalian α1,3/4 FucTs (Fig. 2-5) (Ma *et al.*, 2003)

Removal of the putative α-helices at the C-terminus did not significantly reduce the specific enzyme activity, indicating that the amphipathic helices are not absolutely required for conferring activity. Remarkably, the truncation greatly increased protein expression and protein solubility (Fig. 4-2), which facilitated the purification of a significant amount of soluble active enzyme for detailed kinetic characterization and key polar group mapping. Previously, similar increases of protein expression have been

Table 4-5. Selected data of ^{1}H NMR for feature peaks of disaccharide substrates (Type II and Type I) and newly formed trisaccharides (Lewis X and Lewis A) in *H. pylori* α1,3/4 FucT-mediated enzymatic synthetic reactions. The ^{1}H-chemical shifts (δ) and the coupling constants are given. -: Atom not observed

Compound	**H** $_{\text{Fuc-1}}$	**H** $_{\text{Gal-1}}$	**H** $_{\text{GlcNAc-1}}$	**H** $_{\text{Fuc-5}}$	**H** $_{\text{Fuc-6}}$
Lewis A	5.004 ppm	4.472 ppm	4.506 ppm	4.849 ppm	1.177 ppm
	d, J=3.8 Hz	dd, J=7.6,1.1 Hz	d, J=8.5 Hz	q, J=6.6 Hz	d, J=6.6 Hz
Type I	-	4.415 ppm	4.533 ppm	-	-
		d, J=7.7 Hz	d, J=7.9 Hz		
Lewis X	5.096 ppm	4.444 ppm	4.519 ppm	4.823 ppm	1.178 ppm
	d, J=4.0 Hz	d, J=7.8 Hz	d, J=8.1 Hz	q, J=6.6 Hz	q, J=6.6 Hz
Type II	-	4.458 ppm	4.507 ppm	-	
		d, J=7.8 Hz	d, J=8.0 Hz		

observed by deleting the C-terminal putative amphipathic helices in α1,4 galactosyltransferase transferase LgtC from *Neisseria meningitides* (Persson *et al.*, 2001) and the sialyltransferase CstII from *Campylobacter jejuni* (Chiu *et al.*, 2004). It is worth noting that the increase of protein solubility after removal of the putative amphipathic helices supports their role as a membrane anchor. Nevertheless, a substantial portion of protein was still localized to the membrane fraction when the helices were deleted with or without the preceding heptad repeat region (Fig 4-2C, 4-2D). It is possible that the catalytic domain possesses an additional membrane-attachment region that remains to be identified. Alternatively, protein overexpression may have caused membrane association due to the exposed hydrophobicity of some partially unfolded proteins.

In *H. pylori* FucTs, the putative membrane anchor is connected to the catalytic domain by a series of 2 to 10 heptad repeats (Rasko, 2000), which are thought to act as a dimerization motif (Ge *et al.*, 1997; Martin *et al.*, 1997). Deletion of the entire heptad repeat region (11639^{1-370}, $UA948^{1-371}$, $11639^{0\text{-HepRep}}$) almost completely abolished enzyme activity for both 11639FucT and UA948FucT, suggesting that the heptad repeat region is essential for enzyme activity. Since as few as two heptad repeats have been observed in nature (Rasko, 2000), it is not surprising to observe the full or partial enzyme activity present in the single heptad repeat constructs, $11639^{1\text{-HepRep}}$ and $UA948^{1\text{-HepRep}}$, respectively. Deletion of the putative membrane anchor from the 1-HepRep constructs dramatically decreased the activity of 11639FucT (11639^{1-370}) and completely inactivates UA948FucT ($UA948^{1-371}$) (Fig. 4-1). One explanation is that the flexible stalk region plays a role in positioning the catalytic domain away from the lipid bilayer where the amphipathic α-helices bind, facilitating access to substrates. A stem region with one heptad repeat might be too short and addition of the two amphipathic helices is sufficient to extend the stalk region. Another possible explanation is based on the initial suggestion that the leucine zipper-like heptad repeats mediate dimerization, which is essential for enzyme function (Ge *et al.*, 1997; Martin *et al.*, 1997; Rasko *et al.*, 2000b). So an alternative model can be proposed where the C-terminal amphipathic helices, heptad repeats, and perhaps the catalytic domain all contribute to dimerization, thus full activity is obtained as long as the net interaction energy is high enough to form a stable dimer. The full number of heptad repeats may be sufficient for dimerization therefore the C-terminal putative membrane anchor can be deleted, however, when only one heptad repeat is retained, interaction between the hydrophobic faces of the amphipathic α-helices becomes necessary to aid dimer formation. To assess the plausibility of this model, first experimental validation of the heptad repeats mediating dimerization would be necessary.

A minor doublet band was observed in the FucT expression of constructs 11639^{1-447} and 11639^{1-441} (Fig. 4-2C, Fig 4-3). The nature of this band is currently unknown. Experiments such as two-dimensional gel electrophoresis and the N-terminal sequencing of the band could be pursued to determine its identity.

The dual substrate kinetic analysis shows that *H. pylori* α1,3/4 FucTs possess comparable kinetic parameters to their mammalian counterparts. Specifically, the donor GDP-Fuc K_m values for *H. pylori* α1,3/4 FucTs are similar to those for human FucT III, IV, V (de Vries *et al.*, 1995) and VI (De Vries *et al.*, 1997), and the K_m values for Type II-R acceptor ($R=O(CH2)_8CO_2CH_3$) are similar to that of FucT VI (De Vries *et al.*, 1997). With Type II and Type I acceptors that do not contain the hydrophobic linker (-

O(CH2)$_8$CO$_2$CH$_3$), human FucT III and V display a low k_{cat} ranging from 5.2 min^{-1} to 9.3 min^{-1} (Nguyen *et al.*, 1998). The newly characterized *H. pylori* α1,3/4FucT from strain DMS 6709, which is primarily an α1,4 FucT, possesses a similar acceptor K_m (313 μM) for Type II-R acceptor, a lower K_m (114 μM) for Type I acceptor and a relatively low K_m for donor (5.73 μM). Compared to UA948FucT, DMS6709FucT has 24-fold higher k_{cat} for Type I acceptor (26 s^{-1}) and 16-fold lower k_{cat} for Type II acceptor (0.067 s^{-1}) (Rabbani *et al.*, 2005). It is interesting that the Type II acceptor preference in UA948FucT is due to the 21-fold lower K_m for Type II than that for Type I, but their k_{cat}s are identical; while DMS6709FucT favoring Type I acceptor results from the 388-fold lower K_{cat} but only <3-fold lower K_m values for Type I than for Type II acceptor.

Similar to human FucT V (Qiao *et al.*, 1996), the pattern of the double reciprocal plots for velocity with concentration of either substrate at a series of fixed concentrations of the second suggests that *H. pylori* α1,3/4 FucTs catalyze fucose transfer following a sequential mechanism. The current study cannot distinguish whether the substrate binding is random or ordered, and if it is ordered, whether the acceptor or the donor binds first. In the process of optimizing the purification protocol, it was observed that both 11639$^{1\text{-}441}$ and UA948$^{1\text{-}428}$ bind to the GDP-hexanolamine Sepharose affinity chromatography support column but not to immobilized acceptor-based affinity columns (unpublished data), indicating that *H. pylori* FucTs most likely binds to the donor first, as has been observed for most other glycosyltransferases (Qasba *et al.*, 2005). Further detailed kinetic studies and crystal structures are required to fully resolve this issue.

It has been shown that mammalian α1,3/4 FucTs are able to use 3'-sialylated Type II or Type I acceptors, to synthesize 3'-sialylated-Lewis X or 3'-sialylated-Lewis A, respectively, but with variable efficiencies. The hydroxyl group mapping data demonstrated that UA948FucT tolerates sialylation very poorly. Compared to mammalian α1,3/4FucTs, *H. pylori* 11639FucT and 11637FucT resemble human FucT VI (De Vries *et al.*, 1997) in their preference for neutral Type II acceptor and tolerance for 3'-sialylation, whereas UA948FucT behaves more like human FucT IV and V for its α1,3 activity and α1,4 activity, respectively. Human FucT IV transfers fucose much more efficiently to neutral Type II acceptors than those that are sialylated (Sherwood *et al.*, 2002). FucT V prefers both fucosylated and sialylated Type II acceptor, however it does not favor sialylated Type I structures (de Vries *et al.*, 1995). The α1,3/4FucT from strain DMS 6709 is more like human FucT III as both of them prefer the sialylated Type I over the unmodified Type I acceptor (Rabbani *et al.*, 2005).

Although it appears most *H. pylori* FucTs can utilize the sialylated acceptors, only very few *H. pylori* isolates (2 out 94) were found to express 3'-sialylated-Lewis X (Wirth *et al.*, 1996), such as strain P466 (Monteiro *et al.*, 2000). This is most likely due to the absence of a functional α2,3 sialyltransferase, which has not yet been identified in any *H. pylori* strains. It would be of interest to determine if *H. pylori* α2,3 sialyltransferase is able to use the 2'-fucosylated Type II or 2'-fucosylated Type I as acceptors, like the α2,3 sialyltransferase from myxoma virus (Chiu *et al.*, 2004), or if sialylation has to precede to fucosylation as has been reported for mammalian α2,3 sialyltransferases (Holmes *et al.*, 1986).

The hydroxyl group mapping data show that the 2-fucosylated Type II (H-Type II, **(3)**) is an excellent acceptor for both *H. pylori* FucTs, and 2-fucosylated Type I (H-Type I **(15)**), and it is also a fairly good acceptor for UA948$^{1\text{-}428}$. This challenges the notion that the difucosylated Lewis antigens (Lewis Y and Lewis B) in *H. pylori* are synthesized predominantly via subterminal fucosylation (in α1,3 or α1,4-linkage) followed by terminal fucosylation (in α1,2-linkage) (Wang *et al.*, 2000). Martin's data showed that 11637FucT was unable to use Fuc(α1,2)Galβ1,4Glc as an acceptor (Martin *et al.*, 1997). Since the lack of the *N*-acetamido group in Type II structure severely impaired enzyme activity (Table 4-4), one cannot conclude that 11637FucT is unable to use Fuc(α1,2)Galβ1,4GlcNAc (H-Type II) as an acceptor. The α1,2 FucTs from *H. pylori* were reported to use both the unfucosylated (Type II and Type I) and the fucosylated (Lewis X and Lewis A) structures as acceptors (Rasko *et al.*, 2000a). Therefore, I propose that the difucosylated Lewis antigens in *H. pylori* can be synthesized via either route: α1,3/4 fucosylation followed by α1,2 fucosylation or vice versa. The route used predominantly *in vivo* will depend on activity and expression levels as well as the substrate preferences of α1,3/4 FucTs and α1,2 FucT in a particular *H. pylori* strain. In *H. pylori* strains NCTC11639 and UA948 this is not an issue because their *futC* gene does not encode a functional α1,2 FucT protein (Rasko *et al.*, 2000b; Wang *et al.*, 2000).

The key polar group mapping data show that phosphorylation of the GlcNAc at C-6 position **(8)** caused a dramatic reduction in activity for both enzymes, and the lack of the *N*-acetamido group **(9)** also generated a dramatic decrease in activity, by 250-fold for 11639FucT and by 100-fold for UA948FucT. This implies that the 6-OH and the acetamido group in GlcNAc of Type II acceptor contribute to optimal enzyme activity. It has been previously shown that the acetamido group in Type II acceptor is required for optimal activity of human FucT III, IV and V (de Vries *et al.*, 1995).

The key polar group mapping data suggest that the hydroxyl at C-6 of galactose in Type I and Type II acceptors is a key polar group essential for recognition by *H. pylori* α1,3/4 FucTs (Fig. 4-5). For the reactive hydroxyl group in Type II acceptor, when it is deoxygenated, the compound functions as a weak inhibitor but the relative high K_i value suggests that this hydroxyl does play an important role in enzyme binding. When OH-4 of the GlcNAc moiety in Type I acceptor is deoxygenated, we did not observe any inhibition. As UA948$^{1\text{-}428}$ possesses a relative high K_m for Type I acceptor, the actual K_i is hard to quantify. However, if we assume competitive inhibition and a 5% detection limit of the assay, we estimate the K_i to be 83 mM or greater. A similar sensitivity pattern has been reported for mammalian α1,3/4 FucTs (de Vries *et al.*, 1995; De Vries *et al.*, 1997; Du and Hindsgaul, 1996; Gosselin and Palcic, 1996). This suggests that both mammalian and *H. pylori* α1,3/4 FucTs bind the Type II and Type I compounds in a similar manner. As proposed in Chapter 4, the GlcNAc moiety in Type I structure is rotated by 180° relative to its location in Type II compound, bringing the 4-OH of GlcNAc in Type I to the same position as the 3-OH of GlcNAc in Type II (Ma *et al.*, 2005). The 6-CH_2OH and acetamido groups in Type I are also rotated 180° relative to those in Type II structures. This hypothesis is strengthened by NMR studies of Lewis X and Lewis A trisaccharides, where the fucose and galactose in both compounds occupy very similar relative positions with the major difference being their opposite orientation of the GlcNAc moiety (Azurmendi *et al.*, 2002). We noticed that the absence of the acetamido group **(9)** is the only modification that causes less dramatic effect on the transfer rate observed by UA948FucT compared to 11639FucT (Table 4-4). This suggests that the *N*-acetamido group contributes less to recognition by UA948FucT than 11639FucT. This may at least partially explain why Type I acceptor cannot be properly accommodated by 11639FucT due to the flipped GlcNAc moiety (discussed below).

In conclusion, *H. pylori* α1,3/4 FucTs and their mammalian counterparts seem to share striking functional similarities in spite of a very low level of sequence homology. The functional similarities that have been shown previously with respect to domain architecture and the region and residues to discriminate Type I from Type II acceptors (Ma *et al.*, 2003; Ma *et al.*, 2005) has now been extended to include the minimal catalytic domain, kinetic parameters and key polar groups of acceptors essential for enzyme recognition. The current data suggest that all α1,3/4 FucTs very likely share a conserved mechanistic and structural basis for fucose transfer. This makes *H. pylori* α1,3/4 FucTs an attractive model system for studying the entire FucT family, and the success in purifying soluble, stable, and active truncated forms of *H. pylori* α1,3/4 FucTs allows structural studies to be pursed. In addition, *H. pylori* α1,3/4 FucTs have great potential to be exploited in the enzymatic synthesis of glycoconjugates that contain Lewis blood group structures. Such glycoconjugates are acknowledged to have considerable pharmaceutical value in the prevention of bacterial or viral infection, in the neutralization of toxins, and in immunotherapy for cancer (Dwek, 1996; Endo and Koizumi, 2000; Guo and Wang, 1997; Watt *et al.*, 1997).

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CHAPTER 5

General Discussion

5.1 Lewis Antigens in *H. pylori* Pathogenesis

The initial findings in the 1990's that *H. pylori* LPS contained Lewis blood antigen structures that are also expressed on the gastric surface epithelium and in the deeper glands prompted extensive investigation into the roles of these fucosylated oligosaccharides in *H. pylori* pathogenesis. To date, mounting evidence shows that Lewis blood antigens are not absolutely required for *H. pylori* colonization and adhesion (Altman *et al.*, 2003; Guruge *et al.*, 1998; Suresh *et al.*, 2000; Takata *et al.*, 2002). Instead, they play only a minor role in either process and such modest roles are only observed in some but not all *H. pylori* strains. Nevertheless, the presence of these fucosylated oligosaccharides is often correlated with the occurrence of severe gastric diseases (Eaton *et al.*, 2004; Monteiro *et al.*, 2001; Rasko *et al.*, 2001). It was recently noted that Lewis blood antigens interact with the DC-SIGN ligand (Appelmelk *et al.*, 2003; Bergman *et al.*, 2004) on dendritic cells leading to an increasing amount of IL-10 production. Being a Th2 cytokine, IL-10 blocks the Th1 response and promotes Th2 activation. As a result, the host immune response is modulated (Bergman *et al.*, 2004). Lewis antigen expression is regulated by phase variation at the rate of approximately 0.2-0.5% (Appelmelk *et al.*, 1998; Appelmelk *et al.*, 1999). Therefore, through phase variation, Lewis antigens on *H. pylori* LPS are able to switch from an acute *H. pylori* infection that features predominantly Th1 responses to a chronic infection mediated by a mixed Th1-Th2 reaction. Lewis antigens of *H. pylori* may thereby aid the bacteria in adapting effectively to their niche within the stomach to maintain a persistent infection (Bergman *et al.*, 2004).

5.2 Glycosyltransferases

Glycosylation occurs ubiquitously in biological and pathological processes in almost all organisms. The reaction is catalyzed by a super enzyme family, the GTs. GTs transfer sugars, primarily sugar nucleotide di- or monophosphates, to various targeted acceptors forming glycosidic bonds. The glycosylation reaction can proceed by either inverting or retaining the stereochemistry of the anomeric center of the donor sugar. The mechanism of the former is well understood – a catalytic base in the GT facilitates proton transfer, and aids in the reactive hydroxyl of the acceptor substrate attacking the anomeric center of the donor (Unligil and Rini, 2000). However, the mechanism of the retaining reaction is not clearly defined – the reaction either follows the double-displacement scheme (Davies, 2001; Lairson *et al.*, 2004; Sinnott, 1990; Withers *et al.*, 2002) or a single step nucleophilic attack (Boix *et al.*, 2002; Persson *et al.*, 2001; Tvaroska, 2004). α1,3/4 FucTs are inverting enzymes, as a result a catalytic base in the FucT is expected to be involved in catalysis.

The majority of GTs follow a sequential bi bi mechanism (Charnock and Davies, 1999; Chiu *et al.*, 2004; Gastinel *et al.*, 1999; Patenaude *et al.*, 2002; Persson *et al.*, 2001; Qasba *et al.*, 2005). Specifically, the donor binds to the enzyme first to generate a re-localization of one or two flexible loops, which changes from an open to a closed conformation. Such a conformational change shields the donor from exposure to the solvent and creates a binding site for the acceptor. Nevertheless, at least two members of the retaining GTs are reported to follow the sequential bi bi mechanism, but with acceptor binding to the enzyme preceded to the binding of donor (Quiros and Salas, 1995; Quiros *et al.*, 2000). The kinetic data support that *H. pylori* α1,3/4 FucTs follow the sequential bi bi mechanism (Chapter 4). It remains to be determined if donor or acceptor binds to the enzyme first.

The GT superfamily contains a large number of enzymes, which utilize diverse acceptor molecules for sugar transfer and generate a considerable variety of products. It is therefore expected that the GTs adopt a number of distinctive folds, as is found for the glycosidase family that cleaves glycosidic bonds. However, it appears that the superfamily of GTs adopts a very limited number of folds. The well-studied GT-A (Gastinel *et al.*, 2001; Hu and Walker, 2002; Mulichak *et al.*, 2001; Pedersen *et al.*, 2000; Persson *et al.*, 2001; Tarbouriech *et al.*, 2001; Unligil *et al.*, 2000) and GT-B (Breton *et al.*, 2005; Chiu *et al.*, 2004; Hu and Walker, 2002; Hu *et al.*, 2003; Liu and Mushegian, 2003; Mulichak *et al.*, 2001; Qasba *et al.*, 2005) folds have different active sites and different mechanisms. A new GT-C family fold has been identified recently and the enzymes belong to this group are mainly present in the eukaryotic kingdom (Kikuchi *et al.*, 2003; Liu and Mushegian, 2003; Rosen *et al.*, 2004). In addition, a GT-D fold is proposed as a folding pattern for GTs with unknown characteristics (Liu and Mushegian, 2003). Simulation and structural modeling studies predict that the α1,3/4 FucTs adopt the GT-B fold (Breton *et al.*, 1996; Liu and Mushegian, 2003; Wrabl and Grishin, 2001), while the α1,2 and α1,6 FucT families are grouped into the GT-D fold (Kikuchi *et al.*, 2003).

5.3 Mammalian and *H. pylori* α1,3/4 Fucosyltransferases

The domains of *H. pylori* α1,3/4 FucTs are inverted in comparison to those of mammalian enzymes. This is reflected in the localization of the membrane anchor and the stem region relative to the catalytic domain, the determinant for Type I acceptor specificity and the minimal catalytic domain architecture. Data in this thesis demonstrate that *H. pylori* α1,3/4 FucTs share comparable kinetic parameters with their mammalian counterparts, including the binding affinity to donor and acceptors as well as the K_{cat} values (Chapter 4). Most importantly, the results confirm that these two families of enzymes utilize the same key polar groups in recognizing Type II and Type I acceptors (Chapter 4). These functional similarities strongly suggest that all α1,3/4 FucTs share a common mechanistic and structural basis of fucose transfer. As such, *H. pylori* α1,3/4 FucTs are an appealing model for studying the entire FucT family, especially with the ease of cloning and expression of these enzymes in *E. coli* systems. The ability to purify soluble and stable *H. pylori* α1,3/4 FucTs with a yield of milligrams per liter as shown in Chapter 4 presents an important opportunity to pursue structural determination with the goal of resolving a crystal structure.

5.4 Future Directions

Despite the steady progress that has been made by many investigations to untangle the correlation between Lewis antigen expression and *H. pylori* induced infection and the studies described in this thesis to comprehend the structure-function relationship of *H. pylori* α1,3/4 FucTs, many areas are presently unknown and remain to be examined as discussed below.

5.4.1 Additional Structural-Functional Characterization

The structural characterization of *H. pylori* α1,3/4 FucTs in this thesis has greatly advanced understanding of these enzymes with respect to the Type I acceptor recognition, the kinetic characteristics, and the key polar groups of acceptors that are essential for recognition. Nevertheless, the hypothesis with regards to the functions of heptad repeats and the C-terminal amphipathic α-helices in *H. pylori* α1,3/4 FucTs awaits experimental verification.

5.4.1.1 Heptad Repeats: Dimerization motif

It has been proposed that the heptad repeat region of *H. pylori* α1,3/4 FucTs contain a leucine-zipper like motif to mediate dimer formation (Ge *et al.*, 1997; Martin *et al.*, 1997), however, concrete experimental evidence is lacking. There are two technologies that could be used to resolve this issue and both of them are available at University of Alberta. The first is dynamic light scattering (DLS), which investigates the size distribution of a protein in a dilute aqueous suspension (Bell *et al.*, 1997; Blakey *et al.*, 2002; Pasta *et al.*, 2003; Vanhoudt *et al.*, 2000). The second technology is fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), which utilizes a nano-electrospray as the ionization source (nano-ESI) to convert the protein from liquid phase to gas phase (Campbell *et al.*, 2005; Liang *et al.*, 1991; Reinhold *et al.*, 1995). The tiny droplets produced by electrospray are in a "weak" ionization status, so the resolved conformation of a protein would represent the same configuration in solutions. Both DLS and FTICR-MS could reveal the mass of the protein complex. As a result the aggregation status would be determined.

5.4.1.2 Membrane Association by C-terminal α-helices

A short C-terminal segment of *H. pylori* α1,3/4 FucTs is rich in hydrophobic and positive residues, which meet the requirements necessary to form two amphipathic α-helices. The data show that deletion of these two α-helices increases protein solubility, supporting the predicted function of a membrane anchor (Chapter 4). However, when these two α-helices are deleted and even when the upstream heptad repeat region is deleted, a significant amount of protein is still expressed in the membrane fraction (Chapter 4). This indicates that these two putative α-helices are unlikely the sole determinant for membrane association. To confirm that these two α-helices are indeed involved in membrane tethering, the full-length proteins from *H. pylori* strains 11639 and UA948, the truncated constructs with α-helices removed ($11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$), and the small fragments of the α-helices alone, could be fused with green fluorescent protein (GFP) at C-terminus and their localization determined by fluorescence microscopy. The preliminary data show that both full-length 11639FucT and UA948FucT, when fused to GFP at their C-terminus, remain fully active (B. Ma, unpublished data). This suggests that the GFP fusion does not affect proper protein folding, therefore it is applicable to utilize GFP as a tag to trace the localization of FucT within cell. To identify whether the heptad repeats are also responsible for membrane anchoring, it would be possible to adapt the same strategy as above. Full-length proteins, constructs with the heptad repeat region removed ($11639^{1\text{-}370}$ and $UA948^{1\text{-}371}$) and the heptad repeat region itself could be fused with GFP and localization of FucT enzyme examined by fluorescence microscopy.

5.4.1.3 Donor Binding Sites

This thesis successfully narrowed down the Type I acceptor recognition site of *H. pylori* α1,3/4 FucTs to the C-terminal hypervariable loop region (Chapter 2) and specifically a single aromatic residue within the loop (Chapter 3). The donor binding sites, however, have not yet been defined. A highly conserved Cys residue within the catalytic domain of human α1,3/4 FucTs is confirmed to be the GDP-Fuc binding site (Holmes *et al.*, 1995). Due to low sequence homology among these proteins, it is rather difficult to predict which Cys residue of *H. pylori* α1,3/4 FucT corresponds to the Cys of human enzymes responsible for donor binding. In 11639FucT and UA948FucT, there are four and three Cys residues, respectively (Fig. 2-1), which could be mutated to Ala and then determination made if a substitution abolishes enzyme activity. To confirm whether a Cys is indeed involved in GDP-Fuc binding, chemical treatment by NEM and donor-protection could be carried out. Nevertheless, in *H. pylori* α1,3/4 FucTs, residues other than Cys might bind to the donor, thus chemical modification with reagents that specifically modify Lys, Arg, Trp or His along with donor protection studies might also need to be performed to determine the residues important for donor binding.

5.4.2 Mechanism of *H. pylori* α1,3/4 FucT

5.4.2.1 Substrate Binding Order

The double reciprocal plots derived from the dual substrate kinetic data in this thesis (Chapter 4) show that the *H. pylori* α1,3/4 FucT-catalyzed reaction follows the compulsory bi bi mechanism, however, it is not known whether the donor or acceptor binds the enzyme first. The observation that both 11639^{1-441} and $UA948^{1-428}$ bind to GDP-hexanolamine sepharose supports during affinity chromatography but not to the immobilized acceptor-based affinity column does suggest that *H. pylori* α1,3/4 FucTs most likely bind donor first. To resolve the substrate binding order, competitive inhibitors that mimic the acceptor or donor structures but do not confer enzyme activity could be used to study their inhibitory patterns, with the concentration of one substrate saturating and the concentration of the other substrate varied. The pattern of a series of parallel (uncompetitive inhibition) or intersecting lines (competitive inhibition: same V_{max} and increased K_m, noncompetitive inhibition: same K_m but reduced V_{max}) produced in the double-reciprocal plots determines the substrate binding order. It has been shown that Fuc-T enzymes have a low affinity for the transition state of the GDP-Fuc moiety (Murray *et al.*, 1996) and the affinity for acceptor molecule is also low (De Vries *et al.*, 1997; Miura *et al.*, 1999; Murray *et al.*, 1996). Therefore, it has been difficult to design the acceptor or donor analogues as potent inhibitors (Lee *et al.*, 2003). Nevertheless, GDP-2F-Fuc (K_i=4.2 μM) (Murray *et al.*, 1997) was reported to be a donor competitive inhibitor, and thus can be used in studying the substrate binding order.

5.4.2.2 Metal Cation Requirement

It is not yet known if *H. pylori* α1,3/4 FucTs require metal cations (most commonly Mn^{2+} and Mg^{2+}) for enzyme catalysis. The sequence alignments of *H. pylori* α1,3/4 FucTs shows that three highly conserved EXD motifs are present in all enzymes (Fig. 2-1). The first motif is close to the N-terminus, while the second is within the region of α1,3/4 FucT motif I, and the third is located four amino acids preceeding the hypervariable loop region that confers Type I acceptor specificity. Mutating each "E' or "D" residue within the EXD motifs to Ala by site-directed mutagenesis could be performed. The enzyme activity of each mutant and its sensitivity to the depletion of metal cation could be determined and compared with that of WT. In addition to Mn^{2+} and Mg^{2+}, the effects of other divalent ions, Ca^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+} and Zn^{2+} on enzyme catalysis could also be evaluated. Preliminary data suggest that the metal cations are not absolutely required for the catalysis by 11639FucT and UA948FucT, even though their presence seems to boost enzyme activity (J. Horton, unpublished data). This is consistent with the prediction that α1,3/4 FucTs belong to the GT-B fold enzyme family (Breton *et* *al.*, 1996; Liu and Mushegian, 2003; Wrabl and Grishin, 2001), which generally do not require a metal ion as a cofactor for enzyme catalysis.

5.4.3 Crystallization

The success of purifying soluble and stable *H. pylori* α1,3/4 FucTs with a yield of milligrams per liter would allow crystallization trials to be set up. The current major obstacle for obtaining a crystal is the threshold level (5 mg ml^{-1}) for concentrating the protein solution, where $11639^{1\text{-}441}$ and $UA948^{1\text{-}428}$ still remain soluble. It has been reported that modification of the surface of the target protein, leading to a local reduction of conformational entropy, facilitates crystallization and the formed crystal has superior quality in comparison to those grown from the WT protein (Czepas *et al.*, 2004; Derewenda, 2004a, b). Specifically, hydrophilic residues at the protein surface with large flexible side chains can be mutated to smaller amino acids (i.e. Ala). To facilitate the crystallization procedure, efforts could be made to identify such residues on *H. pylori* α1,3/4 FucTs using bioinformatic tools, and the mutations could be generated to enable crystallization trials to be set up with enzymes having improved crystallization potential.

Provided the difficulty in obtaining a crystal is overcome, it would be of interest to resolve the crystal structure of not only the WT enzymes, but also of several mutants. The structures of constructs $11639^{347CNDAHYSALH}$, $UA948^{345DNPFIFC}$ and $UA948Y^{350}$→A, would provide valuable insights on how the hypervariable loop residues, particularly Tyr^{350}, controls Type I acceptor specificity. In addition, the structure of $11639^{1\text{-}HepRep}$ and $UA948^{1\text{-}HepRep}$ (Chapter 4) could explain how a very short stem region manages to retain full or partial enzyme activity. Moreover, the structures of constructs $11639^{1\text{-}370}$ and $UA948^{1\text{-}371}$ could show why the heptad repeat region is essential for enzyme function.

5.4.4 Identification of *H. pylori* 2,3'-Sialyltransferases and the Role of sLe^x in Pathogenesis

In addition to the expression of Le^x, Le^y, Le^a and Le^b on *H. pylori* LPS (Aspinall and Monteiro, 1996; Aspinall *et al.*, 1996; Aspinall *et al.*, 1997; Chan *et al.*, 1995; Monteiro *et al.*, 1998a; Sherburne and Taylor, 1995; Wirth *et al.*, 1997), sLe^x is also present on the LPS of a very small number of *H. pylori* strains (Monteiro *et al.*, 2000; Wirth *et al.*, 1996). This implies the presence of a 2,3'-sialyltransferase gene in the *H. pylori* genome that has not yet been identified. Twenty distinct sialyltransferases have been identified in human and mouse genomes. Such enzymes transfer the sialic acid from donor CMP-Neu5Ac to the terminal nonreducing positions of the oligosaccharide chains, at α2,3, α2,6, or α2,8 linkages with different acceptor specificities (Harduin-Lepers *et al.*, 2005 and references therein). Despite low sequence identity, these sialyltransferasess share four conserved motifs, motif large (L), motif small (S), motif III, and motif VS (very small) (Harduin-Lepers *et al.*, 2005 and references therein). It would be possible to design several pairs of primers based on the highly conserved sequences within the motif regions. The sialyltransferase gene fragments could then be amplified by PCR using *H. pylori* chromosomal DNA as template, cloned into the vector and sequenced. Since the genome for two *H. pylori* strains is available (Alm *et al.*, 1999; Tomb *et al.*, 1997), a PSI-blast search could determine whether or not the sialyltransferase gene is present in these genomes. If the gene were fully cloned and expressed, it would be interesting to determine the substrate specificity of this enzyme. Moreover, isogenic strains could be generated by knocking out the *H. pylori* sialyltransferase gene. The ability of WT strains and mutant strains to colonize and adhere to gastric epithelial cells could be examined both *in vitro* and *in vivo* in a model system. Sialyl-Le^x is a ligand for E-selectin, which is an endothelial cell adhesion molecule that plays a critical role in the leukocyte–endothelium interaction during inflammation and may contribute to the metastatic dissemination of tumour cells (Hakomori, 1991; Rosen and Bertozzi, 1994; Turner and Catterall, 1997; Varki, 1994). Whether or not the sialyl-Le^x expression on *H. pylori* LPS causes any modification of leukocyte trafficking or causes interaction with any antigen presenting cells to alter host immune response remains to be determined.

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